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In re: deFaire		BOARD OF PATENT APPEALS AND INTERFERENCES
Serial No.:	09/549,642	
Filing Date:	April 14, 2000	
For:	REMOVING DENTAL PLAQUE WITH KRILL ENZYMES	PATENT APPEAL
Docket No.:	314572-101F	
Art Unit:	1642	
Examiner:	Brumback, B.	

APPELLANT'S APPEAL BRIEF

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On the Brief

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(1) Real Party in Interest

The inventors have assigned their interest to Phairson Medical Inc. is the real party in interest.

(2) Related Appeals and Interferences

On information and belief, there are no other appeals or interferences that will directly affect or have a bearing on the Board's decision in this Appeal.

(3) Status of the Claims

Claims 142-144 are in the application. All other claims have been cancelled. All of the pending claims are subject to one or more rejections under 35 U.S.C. §§102(b), 103(a) and 112. Hence, claims 142 through 144 are appealed.

(4) Status of Amendments Filed After Final

There are no amendments after final. The pending claims are attached as Appendix A.

(5) Summary of Invention

The invention rests on the observation that the hydrolases extracted from Krill are very effective in removing plaque. Such an extract was known to be useful as a cleaning agent – but plenty of enzymes used in cleaning are ineffective to remove dental plaque.

(6) Issues

The issues are:

(i) ***Issue 1:*** Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?

(ii) ***Issue 2***: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

(iii) ***Issue 3***: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

(iv) *Issue 4*: Are the phrases "wherein the dental plaque is visually observable" in claim 143 and "until dental plaque is not longer visually observable" in claim 144 supported in the specification?

(7) **Grouping of claims**

The three claims in this application each raise separate issues with respect to one or more of the issues, and shall therefor be argued separately. Accordingly, there is no grouping of the claims.

(8) **Argument**

(i) *Issue 1*: **Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?**

The rejection under 35 U.S.C. §102(b) is premised on principles of inherent anticipation, since an electronic word search of the cited document confirms that not one word relates to any type of plaque. As discussed in M.P.E.P. §2112, such a rejection requires that the element not expressly set forth in the reference necessarily flows from the disclosures of the reference. This requirement is not met here.

The 'Hellgren patent is – in its core discussions – about cleaning wounds and objects such as textiles. One word in the specification mentions that teeth can be cleaned (at 1:31[1]). There is no concrete exemplification from which to infer what type of teeth are to be treated, or even that teeth *in situ* are to be treated. Teeth cleaning occurs often without plaque being effectively removed, or without plaque being present. Thus, treating to effectively remove plaque, as claimed, does not necessarily follow from the brief mention of teeth cleaning in the Hellgren patent.

The most recent Office Action asserts that contacting teeth with the enzymes recited necessarily removes dental plaque. In a sense this is true: If you picked the right amount and

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[1] Column 1, line 31.

application of the enzymes, if you picked teeth that had plaque, you would effectively remove dental plaque as illustrated in the Examples of Appellant's specification. However, the prior art disclosure must teach one what the effective conditions are and what to treat: the cited Hellgren patent does not. Thus, this would only necessarily follow if you made many choices not taught by Hellgren and not necessarily following from Hellgren. Thus, Appellant would submit that this compilation of untaught selection is not what is required to satisfy the "necessarily" standard. Hellgren teaches nothing at all about how much enzyme composition to apply, and does not even teach applying the treatment to teeth that have plaque. So for anticipation, Hellgren is clearly lacking that which would indicate that its methodology (of which, for this dental application, there is none) would necessarily achieve removal of plaque. The rejection is simply not supported by the cited document and should be withdrawn.

The rejection has been extended to claim 143, but nowhere do the Office Actions describe how the cited art teaches selecting subjects where "the dental plaque is visually observable." As the complete disclosure of the cited document is described above, it should be clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

The rejection has been further extended to claim 144, and again the Office Actions do not describe how the cited art teaches that the contacting "is conducted or repeated until dental plaque is not longer visually observable." Since the complete disclosure of the cited document is described above, again it is clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

(ii) *Issue 2*: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

Applicants respectfully submit that the disclosure in Hellgren that krill enzymes can be used to debride wounds or clean textiles does not render the claims obvious. While the results obtained in the debriding and textile cleaning context may be strong, nonetheless enzymes have been used in cleaning textiles for quite some time, and similarly there is a long history of use in

wound debridement. Thus, effectiveness in a different, highly complex environment, namely dental plaque, would not have been apparent to one of ordinary skill.

The complexity of dental plaque is illustrated by the following discussion in US Patent 6,159,447 (a patent which relates to treating plaque with an anchored enzyme):

> Plaque is a heterogeneous mixture of bacterial aggregations embedded in a sticky matrix. While bacterial composition of plaque ranges from 50 to 70 percent, the matrix is derived from dead cells, salivary glycoproteins and serum proteins that are laid on a polysaccharide backbone. The bacteria synthesize the polysaccharides for the plaque backbone as a step in their own colonization process. In addition to the viable bacteria and the matrix, plaque also contains food debris, small numbers of epithelial cells, white blood cells and various other components which are derived from the host and the host's activities.
>
> The formation and development or proliferation of plaque occurs in two stages. The first step may require a base layer of salivary glycoproteins on the tooth's surface as well as on the soft tissue in the oral cavity. This base organic layer, derived from saliva, is adsorbed onto the surface and forms an acquired pellicle. This insoluble acquired pellicle serves as the foundation for supragingival plaque. The second step is the bacterial colonization by "pioneering" bacteria of the acquired pellicle. Once the bacteria have attached to the surface of a structure, they aggregate, develop colonies and plague begins to form.
>
> There are well over 100 different bacterial species in various dental plaques. This variation in the types of bacteria is influenced by diet, salivary components and bacterial interactions, to name a few. The location of the plaque in the oral cavity, the time of the day, age of the patient and the status of the general oral hygiene of the patient all contribute to the implications and consequences of dental plaque and periodontal disease. Consequently, it is not surprising that plaque is a heterogeneous collection of bacterial communities attached to the tooth providing a vast array of biochemical and physiological consequences. Two major pathological conditions as consequences are periodontal disease and dental caries.

> Enzymes as therapeutic agents present unique possibilities. However, some of the early oral pathology research using enzymes was based on the assumption that they would be bactericidal to colonies of organisms found in plaque and therefore would act as "disinfectants". This approach, however, was not fruitful. Recently, it was shown that treatment of buccal epithelial cells with protease altered bacterial adhesion; however, this treatment also distorted the ratios of various bacterial populations. More promising results were obtained when the focus was shifted from bactericidal action to altering plaque formation. These latter results were seen in vitro and in vivo as well as in animal models and humans in clinical trials. However, these approaches also fell short of desired therapeutic effectiveness most likely because the required time for an effective action exceeded the retention time of the enzyme in the oral cavity. In short, salivary flow, other fluid and food movement and normal mechanical agitation in the oral cavity reduced the retention time of the enzyme(s). These factors shortened the residence time of the enzymes, resulting in less than desirable clinical efficacy.

Thus, the above discussion indicates not just that dental plaque is complex, but that other enzymes have apparently failed in removing dental plaque. Further, the publication abstracts attached as Exhibits A-C indicate further that enzyme cleaners can fail to reduce plaque. Accordingly, the cited Hellgren document describes using the enzyme to clean teeth – it does not motivate experimenting to find conditions effective to remove plaque.

Applicant submits that the fact that the type of enzyme mixture recited in the claims would be effective in removing plaque, as illustrated in Examples 33 and 34, and further illustrated in Exhibit D, was obvious only after the Appellant presented the result. Thus, the rejection represents an inappropriate hindsight reconstruction of the invention. Since it would not be apparent that teeth cleaning with the enzymes would produce plaque removal, one would not, absent hindsight, select subjects with visibly apparent plaque so as to satisfy claim 143. It would be further less apparent to continue or repeat the treatment until "dental plaque is not longer visually observable," as required by claim 144.

Accordingly, Appellant submits that this rejection should be withdrawn.

(iii) *Issue 3*: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

Karistam is cited for substantially the same disclosure as is found in Hellgren, but perhaps further including the disclosure that krill enzymes include a hyaluronidase activity that degrades a glycoaminoglycan. Ratcliff is asserted in an Office Action to teach:

> that dental plaque is a complex extracellular matrix containing glucosaminoglycans, chondroitin sulfates, glycoproteins, and proteins. Ratcliff further teaches that degradation of these compounds retards plaque growth.

The March 29, 2001 Office Action cites for these propositions the following text italicized text from Ratcliff:

> Bacterial agglutinigation includes the conversion of sucrose to glucans and fructans by enzymes known as glycosyltransferases. These enzymes are of bacterial origin. The plaque maas [sic] becomes a complex extra cellular (of microorganisms) matrix containing sulphated glucosamineglycans, proteoglycans, glycoproteins, sugar, proteins and lipids which aid in the process of bacterial agglutination. These compounds include the presence of sulphur and become unstable in the presence of high oxygen compounds. The oxygen splits the sulphide bonds to form sulphates or SO_2.
>
> Clinical observations by the inventor have led to the conclusion that **all of these biochemical compounds are attacked to a greater or lesser extent by stabilized chlorine dioxide**. Since these compounds may be used as nutrients for bacteria, the reduction of the compounds will inhibit bacterial growth. More specifically, the stabilized chlorine dioxide oxidizes carbohydrates, chondroitin sulphates, glucosaminglycans, glycoproteins, proteins and lipids. Since these compounds arise as bacterial by products and debris from dead and dying cells, are of salivary origin and are the mechanism of agglutination of the plaque mass, their degradation/oxidation retards plaque growth.

Ratcliff at 3:62 – 4:22 (citation omitted).

Applicant would respectfully submit that Ratcliff indicates that **stabilized chlorine dioxide** attacks the complex mixture of components of plaque. This teaching has no bearing on whether or not Hellgren's composition would be effective. In fact, it teaches that what the Merck Index describes as a "strongly oxidizing" gas that "reacts violently with organic compounds" is required. The rejection is simply in error.

Accordingly, Applicant respectfully submits that the rejection is in error, and should be withdrawn.

(iv) *Issue 4*: Is the phrase "wherein the dental plaque is visually observable" in claim 143 supported in the specification?

Claims 143 and 144 stand rejected under 35 U.S.C. §112, first paragraph, based on an assertion of lack of support in the specification. This support is clear from the two relevant working examples.

The two relevant examples in the specification read:

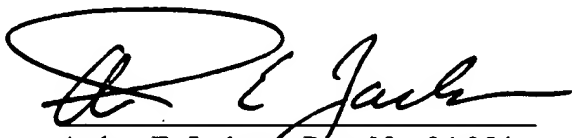
> The krill poly-enzyme preparation was used to remove dental plaque in beagles. Before use, ampoules of the poly-enzyme preparation (described in Example 1A) were reconstituted in 5 ml of saline to a final concentration of 5 Casein-Units/ml. The content from a freshly prepared ampoule was carefully painted over teeth and gingiva. The tongue was fixated for 2 minutes and food and beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily until all plaque was completely decomposed. The dogs were ***inspected*** for status of plaque, saliva secretion and adverse reactions once daily. Eight beagles with abnormal plaque formation due to special feeding and housing were included in this study. After 4 days ***all signs of plaque*** were gone and the study was terminated. No adverse reactions could be observed.
>
> The krill poly-enzyme preparation was used to remove dental plaque in humans. One ampoule of poly-enzyme preparation of Example 1A was reconstituted, in 5 ml of saline to a final concentration of 5 Casein-Units/ml, before each treatment and used to rinse the patient's mouth cavity for 5 minutes. Food and

> beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily and the patients were ***inspected once daily for plaque***, saliva secretion, dryness, and adverse reactions. The patients were not allowed to brush their teeth during the study period. The treatment was continued until ***all signs of plaque*** were gone, but not for longer than 7 days. Two hours after the first treatment all patients experienced a soft and smooth sense over their teeth but ***visual inspection showed remnants of plaque***. Two hours after the third treatment, ***all signs of plaque were gone*** and treatments were terminated. No adverse reactions were observed.

From the above, it should be quite clear that the subjects had visually apparent signs of plaque, and that appropriate treatment can be conducted to remove such visual signs. Accordingly, the specification supports the questioned recitations. Therefore the rejections should be withdrawn.

CONCLUSION

For the foregoing reasons, Appellant respectfully requests that the rejections under 35 U.S.C. §§ 102, 103 and 112 with respect to all of the pending claims be reversed and the pending claims in the application allowed.



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Dated: August 12, 2002

Appendix:

Claims on Appeal.

Exhibits:

A: Abstract of J. Clin. Periodontol 7:431-42, 1980.
B: Abstract of J. Clin. Periodontol 16:295-9, 1989.
C: Abstract of Community Dent. Oral Epidemiol. 3:271-5, 1975.
D: Berg et al., "Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control I," European J. Oral Sciences 109:316, 2001.

APPENDIX A - COPY OF CLAIMS ON APPEAL

142. A method of removing dental plaque in an animal subject comprising: contacting the dental plaque with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

143. A method of removing dental plaque comprising:
contacting the dental plaque, wherein the dental plaque is visually observable, with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

144. The method of claim 143, wherein the contacting is conducted or repeated until dental plaque is not longer visually observable.

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1: J Clin Periodontol 1980 Dec;7(6):431-42 Related Articles, Books, Link

Chemical inhibition of plaque.

Hull PS.

Attempts to control plaque by chemical means using enzymes, antibiotics an antiseptics are reviewed. Enzymes such as mucinase, dehydrated pancreas, enzymes of fungal origin, dextranase and mutanase showed limited clinical success despite promising in vitro and animal studies. Side effects from the of enzymes were observed. Many antibiotics have been used in attempts to control plaque and several have been successful. However, problems exist from the long-term use of such drugs which precludes their routine use as agents for controlling plaque. The biguanide chlorhexidine is the most widel used and investigated method of chemical plaque control. Many studies hav been demonstrated that it will successfully control plaque. No toxic side effects have been reported from its long-term use but local side effects such staining of the teeth do occur. The quaternary ammonium compounds have a present no advantages over the biguanides and require more frequent usage t achieve the same degree of plaque control as chlorhexidine.

Publication Types:
- Review

PMID: 7012186 [PubMed - indexed for MEDLINE]

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B

1: J Clin Periodontol 1989 May;16(5):295-9 Related Articles, Books, Link

Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpas on the development of plaque and gingivitis.

Moran J, Addy M, Newcombe R.

Department of Periodontology, Dental School, University of Wales College Medicine, Heath Park, Cardiff, UK.

Many toothpastes have been formulated over recent years to contain antimicrobial compounds with the aim of preventing or reducing plaque, calculus, gingival inflammation or dental caries. For many, if not all of these toothpastes, it has yet to be proven whether they are significantly better at reducing plaque and gingivitis than conventional toothpastes, for which no such therapeutic effects have been claimed. This 12-day, incomplete block designed, cross-over study compared the development of plaque and gingivi following rinsing with toothpaste slurries containing the following active ingredients: (1) hexetidine/zinc citrate, (2) 0.2% triclosan, (3) amyloglucosidase/glucose oxidase, (4) sodium fluoride/sodium monofluorophosphate (NaF, MFP). By the 8th day of the study, a significant difference in gingival crevicular fluid (GCF) and GI was found between the groups. By day 12, however, no significant difference in plaque index and gingival inflammation was found between the 4 toothpastes, although plaqu area was significantly reduced with the hexetidine/zinc citrate paste when compared to the conventional fluoride paste. It was concluded that the active ingredients added to the toothpastes evaluated in this study provided little or no more additional benefit to oral hygiene and gingival health than could be achieved with a conventional fluoride toothpaste.

Publication Types:
- Clinical Trial
- Controlled Clinical Trial

PMID: 2498397 [PubMed - indexed for MEDLINE]

1: Community Dent Oral Epidemiol 1975 Nov;3 (6):271-5

Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults.

Robinson RJ, Stoller NH, Vilardi M, Cohen DW.

One hundred and thirty-one young adults were used in a controlled study to determine whether a proteolytic mouthwash produced from B. subtilis could help remove existing plaque, decrease the rate of plaque accumulation or reduce the clinical signs of gingivitis. These subjects were stratified by mea of the Gingival Index and the Shaver-Schiff Plaque Index into severe or mil groups. These groups were further divided in a random fashion into treatme and placebo groups. The treatment group rinsed with a proteolytic mouthwa consisting mainly of neutral and alkaline proteases and amylase. A placebo mouthwash was used by the control group. The results of this investigation indicate that there is no statistically significant reduction either in existing plaque or in the rate of plaque accumulation when this proteolytic enzyme mouthwash is used. Furthermore, in the treatment group the degree of gingivitis as measured by the GI was not reduced.

Publication Types:
- Clinical Trial
- Randomized Controlled Trial

PMID: 1104247 [PubMed - indexed for MEDLINE]

Full Article

View/Print PDF article (493K)

European Journal Of Oral Sciences
Volume 109 Issue 5 Page 316 - October 2001

Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control

I. Cecilia Hahn Berg [1], *Sotirios Kalfas* [2,3], *Martin Malmsten* [1,4], *Thomas Arnebrant* [1,5]

This paper deals with enzymatic removal of dental plaque, *in vitro* as well as *in vivo*, using proteases from the Antarctic krill shrimp (*Euphausia superba*), referred to as Krillase®. Krillase exhibits both endo- and exopeptidase activity but has no microbicidal effect. In model systems with pure cultures of oral microorganisms, Krillase demonstrated inhibition of microbial adhesion to saliva-coated hydroxyapatite. Furthermore, a protocol for the growth of reproducible *in vitro* plaque films has been developed, and effects of Krillase on the plaque film were investigated by means of scanning electron microscopy (SEM). The results showed that Krillase efficiently released microorganisms from plaque *in vitro*, the effect being dependent on the enzymatic activity. The surface energy of the substratum had a minor influence on the formation and removal of plaque *in vitro*. Ellipsometric studies on the formation and enzymatic removal of a salivary pellicle indicated that the enzymatic effect on plaque may partly depend on degradation of the salivary pellicle. Krillase was also able to remove plaque accumulated on dentures *in vivo*. Our results demonstrate the potential of Krillase for plaque control, and that these enzymes are worthy of further investigations including clinical studies and work to find a suitable vehicle.

Accumulation of microbial plaque on tooth surfaces is the cause of caries and periodontal diseases, the most prevalent oral disorders that affect industrialised societies and account for the considerable research interest paid to the subject during the last decades. Plaque development proceeds through various phases (1-4). Initially, salivary proteins such as acidic proline-rich proteins, statherin, histatins, amylase and mucins adsorb rapidly to the solid surface and form a salivary pellicle (5, 6). The pioneer bacterial colonisers adhere to the pellicle within the next couple of hours, employing various strategies ranging from non-specific physicochemical attachment (7) to recognition of specific receptor sites at the salivary proteins and using cell surface fimbriae (8-11). Other species co-adhere with the primary colonisers, and a complex microbial community is then developed through growth and stratification of the bacteria embedded in a matrix of polymers of bacterial and salivary origin.

Control of plaque accumulation can be achieved by mechanical methods such as brushing and flossing, and by use of chemical substances such as surfactants and antibiotics (12-17). If done properly, mechanical means will give adequate results, but there are also occasions when chemical plaque control is advantageous as a complement in order to counteract plaque formation. The main disadvantage of the latter methods is that the chemicals may selectively inhibit different populations of

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bacteria, resulting in disturbances of the balance in the oral ecosystem. Emergence of resistant bacterial clones upon frequent use of antimicrobial substances is another factor limiting extensive use of some anti-plaque agents.

Enzymes with a broad spectrum of specificity for bacterial surface structures and polymers in the extracellular matrix of dental plaque have also been tried for chemical control of plaque (18). These agents usually lack the above disadvantages and have comparably low antimicrobial profile. The efficacy of enzyme formulations in removing dental or denture plaque varied in clinical studies (19-27). The substrate specificity of the enzymes tested and the administration methods employed have certainly influenced the results in the different studies.

A natural mixture of digestive proteases (referred to as Krillase®) extracted from the Antarctic krill shrimp *Euphausia superba* was earlier characterized and tested for debridement of ulcerative lesions (28, 29). Krillase consists of endo- and exopeptidases able to degrade complex proteinaceous substrates more efficiently than single-enzyme preparations. In addition, the enzymes in Krillase have naturally been selected to co-exist, which makes the autolysis rate of Krillase low. These specific properties of Krillase provided the incentive to conduct this study to examine the effect of Krillase on bacterial viability and adhesion to saliva-coated surfaces as well as the ability of Krillase to counteract formation of salivary pellicle and microbial plaque.

In order to study events at the molecular level, ellipsometry was employed. Based on the measurement of polarisation changes of light upon reflection, ellipsometry enables monitoring of adsorption/desorption processes *in situ* with a time resolution in the order of seconds (30).

Material and methods Go to: Choose

Ch micals

Tris (tris[hydroxymethyl]aminomethane, ≥99% purity) and bovine serum albumin (≥96% purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). (3, 3-dimethylbutyl)dimethylchlorosilane (97% purity) was from ABCR (Karlsruhe, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). The water used in the *in vitro* plaque and ellipsometry experiments was treated by a Milli-Q Plus unit (Millipore, Bedford, MA, USA) including ion exchange, active carbon adsorption and reverse osmosis before the final 0.22- μ m filtration step, yielding ultrapure reagent-grade water of resistivity 18.2 MΩcm at 25°C. In all the other experiments, de-ionised water was used. Ethanol (99.7% pure) was from Primalco (Helsinki, Finland) and Krillase was obtained from BioPhausia (Uppsala, Sweden) (batch numbers B038912, 610008, 604040 and 710017). The lyophilised preparation was reconstituted in water (2 ml/vial) and further diluted with 0.1 M Tris-HCl buffer, pH 7.5, to obtain the appropriate concentration (total proteolytic activity as determined by the supplier and expressed in units per ml solution, U/ml). The characteristics of the enzyme mixture have been reported elsewhere (28, 31-34).

Effect on bacterial viability

Streptococcus mutans NCTC 10449, *Streptococcus sanguis* ATCC 10556, *Actinomyces naeslundii* ATCC 12104, and *Candida albicans* CHR (own isolate) were grown at 37°C for 24 h in the presence of 5% CO_2 on *Brucella* agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% lysed blood, 0.05 mg/ml hemin and 0.01 mg/ml vitamin K. Cells harvested with a sterile cotton swab were suspended in sterile A-buffer (1 mM sodium phosphate buffer containing 50 mM KCl, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.0) and the optical density (at 600 nm) of the suspension was adjusted to 0.5.

Cell suspension (final density 10^6-10^8 cells/ml) was mixed with Krillase solution (final activity 1 U/ml) and A-buffer to a final volume of 1 ml. In the controls, the

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Image Previews

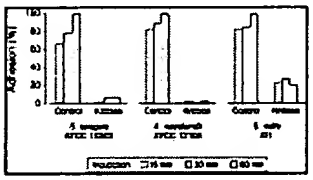

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Fig. 1 Relative (%) number of bacteria adherent to saliva-coated hydroxyapatite beads at different inc...

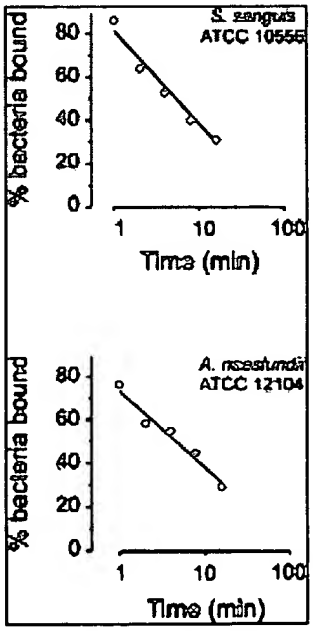

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Fig. 2 Kinetics for Krillase-dependent (activity 0.5 U/ml) detachment of *S. sanguis* and *A. naeslundii*... ...

Krillase solution was replaced by A-buffer. The assays were run aerobically at 37° C. Samples were taken from the mixtures after 15, 45 and 75min of incubation and diluted serially in cold 0.1 M potassium phosphate buffer (pH6.8). From appropriate dilutions, 0.1-ml aliquots were inoculated on *Brucella* blood agar plates and incubated for 48h at 37°C in 5% CO_2. The number of viable bacteria in the samples was determined from the number of colonies grown on the plates and is expressed in colony-forming units (CFU) per ml.

Effect on bacterial adhesion to saliva-coated hydroxyapatite

Streptococcus mitis St1 (own isolate), *S. sanguis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, and *A. naeslundii* ATCC 12104 were grown in Trypticase soy broth (Becton Dickinson) supplemented with 0.5% yeast extract (Difco, Detroit, MI, USA) and 5μCi/ml [^{3}H]thymidine (Amersham, Little Chalfont, Bucks., England) as described above. Cells were harvested by centrifugation (6000× *g* at 6°C for 10 min) and washed twice with 5ml A-buffer before suspended in A-buffer to yield an optical density of 2.0 at 600nm.

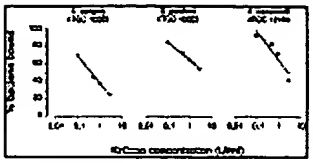
Porous hydroxyapatite beads (lot number 011093-01; Clarkson Chromatography Products, South Williamsport, PA, USA) were coated with paraffin-stimulated whole saliva (SHA beads). The saliva was freshly collected on ice and clarified by centrifugation (16,000× *g*, 6°C, 10min) before used. Alkali-washed hydroxyapatite beads were rehydrated with A-buffer overnight before incubation with saliva (40mg beads/ml saliva) at 6°C under rocking for 1h. The saliva was removed, and the beads were washed with 2×2ml of A-buffer followed by a 30-min incubation with A-buffer containing 0.5% bovine serum albumin. Finally, the beads were washed with 3×3ml of A-buffer.

Cell suspension (final density about 10^8 cells/ml) was mixed with 40mg SHA beads and Krillase solution (final activity 1U/ml). Sterile A-buffer was added to a total volume of 1ml. The enzyme solution was replaced by pure A-buffer in the controls. The mixtures were incubated aerobically at 37°C under continuous rocking. After 15, 30, and 60min of incubation, the supernating suspension was removed, and the beads were washed with 3×1ml of A-buffer containing 0.1% formalin in order to stop the enzymatic activity and remove non-adherent cells. The beads were thereafter mixed with 10ml of scintillation cocktail (Biosafe; Beckman, Fullerton, CA, USA) and the radioactivity from bacteria adherent to the beads was counted in a β-counter (Rackbeta; LKB Wallac, Turku, Finland). The number of bacteria adherent to the beads was calculated from the radioactivity of the samples. Relative adhesion is given as a percent of the maximum value obtained in the control mixture at 60min.

In order to examine the effect of incubation time and enzymatic activity on the anti-adhesive properties of Krillase, cell suspension (final density about 10^8 cells/ml) was mixed with SHA beads (1ml suspension to 200mg beads) and incubated aerobically at 37°C for 45min under continuous rocking. The cell suspension was removed, and the beads were washed twice with 2ml of A-buffer in order to remove non-adherent cells. Krillase solution (0.5ml, 0.5U/ml) was added, and the mixture was incubated at room temperature for defined periods of time. In separate experiments, Krillase at various activities was added to the beads, and the mixture was incubated for 5min. The assay was terminated by the addition of 1ml 2% formalin in A-buffer. The suspension was removed, and the beads were washed twice with 2ml A-buffer in order to remove non-adherent cells. The beads were thereafter mixed with 0.5ml 1M KOH and incubated for 10min before the solution was neutralised with 1M HCl. The beads and the liquid phase were mixed with 10 ml of scintillation cocktail, and the radioactivity was counted as described above. Relative adhesion is given as percent of the maximum value obtained in the control mixtures (no enzyme added or 0min incubation time).

Effect on plaque formed *in vitro*

In order to obtain *in vitro* plaque, saliva samples obtained after gustatory stimulation with a sugar lump were poured into polystyrene Petri dishes (about 8ml



[Full Size]

Fig. 3 Effect of enzymatic activity on the Krillase-dependent detachment of *S. sanguis*, *S. gordonii* a...

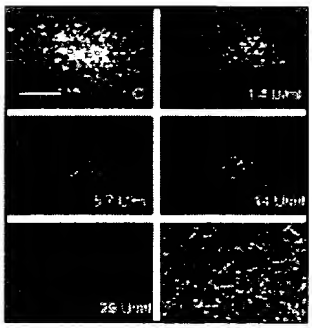


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Fig. 4 SEM images of *in vitro* plaque on glass slides incubated for 15min in Tris-HCl buffer (control ...

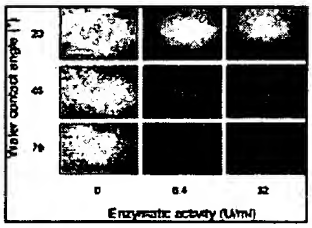

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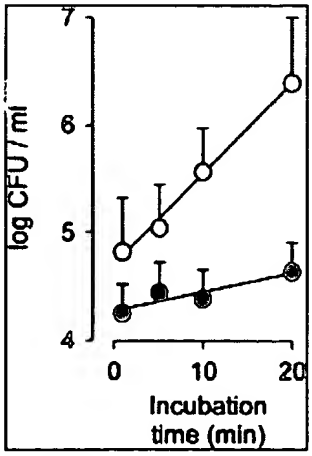
Fig. 5 SEM images of *in vitro* plaque on glass slides with various surface energy, exposed to either T...



[Full Siz]

saliva per dish) containing glass slides ($1 \times 1 cm^2$ each) that had been rinsed with water and ethanol and air-dried. The dishes were incubated at 37°C for 48h. The plaque formed on the slides was rinsed twice with water, and excess water was absorbed carefully from the slide edges. The slides were then immersed for 15min in dishes containing warm (37°C) solution of Krillase in 0.1M Tris-HCl buffer, pH 7.5, or buffer alone (control). The samples were rinsed twice with water and air-dried. The sample surfaces were coated with a thin layer (~90nm) of Au/Pd (2 ×180s, 40mA; Balzers Sputter Coater SCD 050) and then examined with a Philips scanning electron microscope (SEM 515) operating at 15kV.

In separate experiments, the influence of surface energy of the substratum on plaque formation and removal was studied. For this purpose, the glass slides used for plaque accumulation had been boiled (80°C) for 5min in a 1:1:5 (by volume) mixture of 25% NH_3, 30% H_2O_2 and water, followed by rinsing in water and a second boiling step as above. Finally, the slides were rinsed twice with both water and ethanol. This treatment yielded hydrophilic glass surfaces with a static water contact angle of 20°, as measured by goniometry. From the hydrophilic glass surfaces, hydrophobic surfaces of two different surface energies were prepared by means of liquid phase silanisation in either p-xylene or trichloroethylene (35, 36). After rinsing in ethanol and p-xylene or trichloroethylene, the slides were immersed in either 0.1% or 0.06% solutions of (3, 3-dimethylbutyl)dimethylchlorosilane in p-xylene or trichloroethylene for 15 or 90min, respectively. The slides were finally rinsed four times with p-xylene or trichloroethylene and four times in ethanol and then stored in ethanol. These procedures yielded surfaces with static water contact angles of 46° (p-xylene solution) and 79° (trichloroethylene solution).

Effect on plaque formed *in vivo*

Fifteen subjects wearing dentures and having a clinically healthy oral mucosa participated in the study. All participants obtained detailed information about the study. The participants visited the clinic on three occasions. On the first visit, the dentures were cleaned professionally with brush, water and detergent. The participants were thereafter instructed to wear their dentures as usual for the consecutive 2d and to refrain from any denture cleansing procedure, apart from rinsing with tap water after meals to remove food remnants. On the third day, the plaque-removing ability of Krillase was tested. Each denture was immersed in 500 ml of water and kept under gentle agitation on a magnetic stirrer for 1min in order to remove saliva and loosely attached food remnants. The denture was then immersed in 120ml of Krillase solution (1 U/ml) and incubated at 25°C for up to 20 min under gentle agitation by orbital rotation at 18rpm. At defined time points, samples were aspirated and diluted with 0.1M potassium phosphate buffer containing 0.5% NaCl (pH6.8). From appropriate dilutions, 0.05-ml aliquots were inoculated on *Brucella* blood agar plates. The plates were incubated aerobically at 36°C for 48h, and the total number of colonies was counted.

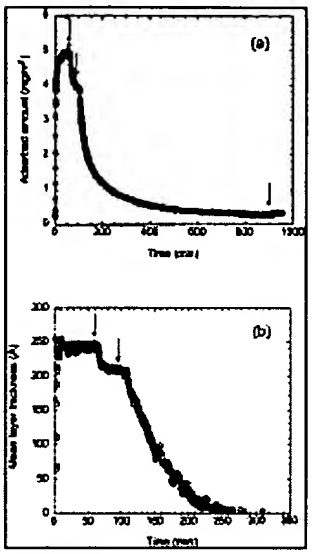
One week later, the subjects visited the clinic again having allowed plaque to accumulate on the dentures for 2d as outlined above. The assay for plaque removal was repeated, but on this occasion a heat-inactivated (20min in boiling water) Krillase solution was used to serve as a negative control.

Effect on pellicle formation

Silica slides (Si/SiO_2) used for ellipsometric experiments were oxidised and cleaned as described in detail elsewhere (37). The silica surfaces were hydrophilic with a static water contact angle of <10°. Prior to usage, the slides were stored in ethanol. The silica surfaces were plasma-cleaned for 5min immediately before the ellipsometric measurement. Cleaning was performed in low-pressure residual air using a radio frequency glow discharge unit (Harrick Plasma Cleaner PDC-3XG; Harrick Scientific, Ossining, NY, USA).

Adsorption of salivary proteins to silica surfaces was followed by null ellipsometry, using an automated Rudolph Research thin film ellipsometer (type 43603-200E; Rudolph Research Analytical, Flanders, NJ, USA), controlled by a personal computer and operated at an angle of incidence of 67.7°. A xenon lamp, filtered to

Fig.6 Release kinetics of microbes from dentures immersed in Krillase solution (1 U/ml, open symbols...



[Full Size]

Fig.7 Kinetics for salivary pellicle formation and enzyme-induced removal, as determined *in situ* by ...

To cite this article:
Hahn Berg, I. Cecilia, Kalfas, Sotirios, Malmsten, Martin & Arnebrant, Thomas
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4015 Å, constituted the light source. The silica surface was mounted in a thermostated (25°C) quartz cuvette, agitated by a magnetic stirrer at about 90rpm. Each experiment was preceded by a four-zone calibration of the surface in two ambient media, air and buffer (10mM sodium phosphate buffer with 50mM NaCl, pH7.0), in order to determine the complex refractive index of the silicon and the refractive index and thickness of the oxide layer. A detailed description of the instrumental set-up and procedure can be found elsewhere (38). Unstimulated saliva, collected according to the procedure described by DAWES (39), was added to the buffer in the cuvette to a final concentration of 10% (v/v). The adsorption process was monitored continuously in one zone, taking into account the corrections for imperfections in optical components obtained from the four-zone calibration. In order to remove non-adsorbed salivary components, the pellicle formation was interrupted after 1h by rinsing with buffer for 35min. Krillase was then added to a final activity of 0.14U/ml, and following the degradation process (after 13h and 25min), the cuvette was finally rinsed for 45min. The flow rate used at rinsing was 1.6ml/min.

The thickness and mean refractive index of the adsorbed film are related to the output parameters of the ellipsometer, the ellipsometric angles Ψ and Δ. The adsorbed amount per unit area can be calculated (40), if the refractive index increment of the adsorbing molecules with concentration in solution (dn/dc) is known and assumed to be constant up to the concentration found in the adsorbed layer. The value of dn/dc has been found to show small variations for different types of proteins, and values of about 0.18ml/g have been measured, and applied, within a broad concentration range for several other proteins (40). This value was therefore chosen as a reasonable approximation for salivary proteins. Knowledge of the partial specific volume (ν) and the ratio of the molar weight to the molar refractivity (M/A) of the adsorbate permits the adsorbed amount per unit area to be calculated by applying another model (41). The values of ν and M/A employed here, 0.75ml/g and 4.10 g/ml, respectively, are representative for proteins, and have previously been used in studies of salivary protein adsorption (42, 43). For the sake of comparison, both models were applied (although only the results from the model according to DE FEIJTER *et al.* (40) are shown) giving qualitatively the same results. Minor quantitative discrepancies can be ascribed to the choice of approximate values of ν, M/A and dn/dc.

Results Go to: Choose

Incubation of *A. naeslundii, S. sanguis, C. albicans* and *S. mutans* with 1U/ml Krillase for up to 75min had no effect on the bacterial viability, the counts of viable cells in the suspension remaining the same as in the enzyme-free controls (results not shown).

Presence of Krillase decreased the adhesion of *A. naeslundii, S. mitis* and *S. sanguis* to SHA beads by ≥70% (Fig. 1). The highest binding to the beads was observed in the controls, and the binding increased with the incubation time. The decrease in adherent cells varied with the strain, and most of the effect was reached within 15min.

Detachment of oral bacteria from SHA beads by Krillase depended on the incubation time (Fig.2) and enzymatic activity (Fig.3). The higher the Krillase activity, the shorter the time needed to remove bound cells. The rate of removal for the three strains tested seemed to be similar (Fig.3). In general, 1.0U/ml Krillase removed approximately 50% of the cells within 5min under the present experimental conditions.

Fig.4 shows the effect of Krillase on *in vitro* plaque as observed by SEM. Compared to the enzyme-free control, Krillase reduced the adhered microorganisms, the effect being dependent on the enzyme activity of the solution. Most of the bacteria in the artificial plaque were rod-shaped, as can be seen in the image captured at higher magnification.

The formation and enzymatic removal of *in vitro* plaque were examined with surfaces of various wettabilities. The results (Fig.5) showed that the lower the water contact angle of the surface, the denser the bacterial accumulation. No major influence by the surface wettability could, however, be observed on the plaque removal by Krillase.

The release of bacteria from denture plaque (Fig.6) proceeded with a rate of about 10^5 cells/min in the presence of 1 U/ml Krillase. This rate appeared to be constant within the time period tested (20 min). For heat-inactivated Krillase, a much slower detachment rate was found (Fig.6).

In order to collect information on the mechanisms for the enzymatic effect on plaque removal, the formation and subsequent enzymatic degradation of a salivary pellicle by Krillase on hydrophilic silica was monitored *in situ* by ellipsometry. The kinetics of the amount of protein adsorbed and the changes in adsorbed layer thickness are shown in Fig.7. The initial adsorption of salivary proteins was fast, and the adsorbed amount reached a plateau value of about 5 mg/m^2 after less than 1 h. Rinsing with buffer (at 60 min) removed reversibly bound proteins, and the adsorbed amount as well as the thickness decreased accordingly and reached new plateau values. Addition of Krillase (at 95 min) induced a gradual decrease in adsorbed material almost immediately. The process was most pronounced during the first couple of hours, then gradually slowed down. The residual adsorbed amount after 14 h of degradation was 0.4 mg/m^2. This layer was not affected by the final rinsing with buffer (at 900 min). When the adsorption of Krillase alone was tested, the plateau value of the adsorbed amount to the silica surface was about 0.2 mg/m^2 (results not shown).

Discussion Go to: Choose Go

It is evident from the present data that the enzyme mixture Krillase is capable to inhibit adhesion of oral bacteria to saliva-coated surfaces and also to detach bacterial plaque formed *in vitro* or *in vivo* on these surfaces. Thus, Krillase appears to exhibit two main mechanisms whereby plaque formation can be controlled: 1) by reducing the accumulation rate of new plaque and 2) by removing existing plaque (18). Furthermore, Krillase exhibits no microbicidal effect and, thereby, it could fulfil another important criterion for plaque control chemicals, namely to maintain an undisturbed relative balance of individual microbial species in the oral ecosystem.

The mechanism behind the inhibition of plaque accumulation appears to rely on the enzymatic degradation of salivary and microbial proteins involved in adhesion-mediating interactions between microbial cells and saliva-coated surfaces. The ability of Krillase to remove a salivary film was clearly demonstrated by the ellipsometry experiments with saliva-coated silica surfaces that were exposed to Krillase solution. Ellipsometric measurements require a highly reflective and smooth substrate surface, such as the silica surface chosen in this study. Since the surface chemistry of silica and glass is virtually the same, this also enables a comparison with results obtained with glass as substrate. Upon exposure of an adsorbed protein layer to proteolytic enzymes, various effects can be anticipated, including competitive adsorption, degradation and interfacial exchange phenomena. The present experiments revealed that Krillase, on a clinically relevant time scale (min), significantly reduced the thickness and amount of salivary proteins adsorbed to the silica surface, indicating a modification of domains/peptide segments involved in bacterial attachment.

Inhibition of plaque accumulation through destruction of microbial adhesins was not shown directly in this study. However, support for such an effect was found in earlier experiments where Krillase treatment of plaque suspensions resulted in a significant decrease in microbial cell hydrophobicity and co-aggregation pattern (own unpublished data). Further support for a Krillase-dependent effect on bacterial adhesins is derived from the experiment with the denture plaque. This plaque was accumulated for 2-3 d and, thereby, it reached a thickness of several bacterial layers. Considering the plaque architecture, the action of Krillase can be assumed to occur on the superficial bacterial layer, which has to be removed before the

enzymes will degrade the next more deeply situated layer. Thus, removal of denture plaque by Krillase implies the ability of the enzyme to destroy co-aggregates of plaque bacteria, which are mainly formed by specific adhesin-receptor interactions known to bind various oral bacteria to each other (44).

The adhesion-counteracting effects of Krillase were time dependent and activity dependent in all the experiments with pure bacterial cultures and plaque formed *in vitro*. The bacterial deposits studied in the *in vitro* experiments are mainly monolayers adherent to the saliva-coated surfaces and differ from *in vivo* formed plaque. To simulate the *in vivo* situation, experiments were included with plaque formed on dentures. This plaque was also removed in a time-dependent manner, albeit at a rate that may be considered as slow from a clinical point of view. Finding the optimal Krillase activity for clinical applications may be one of the future goals. Efforts may also be focused on developing formulations of the enzyme with increased bio-availability, since the velocity of the enzymatic reaction is expected to be a function of the total surface area of the substrate and the area occupied by the enzyme (45).

Glass and silica surfaces served as simple models of a tooth surface in this study. In some experiments, the basic requirement on the model surface was a minimal surface roughness. Initial investigations were made with sintered hydroxyapatite discs, but the porous nature of this material made interpretation of the results difficult. Replacing hydroxyapatite by glass enabled distinguishing bacterial deposits from the smooth background of the glass surface in SEM analysis. Still, it must be kept in mind that the tooth surface is not as smooth as glass, and that an influence of surface roughness on the formation and retention of plaque can be anticipated (46).

Previous studies have shown that the degree of hydrophobicity of the substrate surface influences the adsorption of salivary proteins, hydrophilic surfaces giving less adsorption, both *in vitro* and *in vivo* (42, 47). Surface wettability also affects the adhesion of oral bacteria, and hydrophobic surfaces have been found to accumulate less plaque *in vitro* as well as *in vivo* (46, 48, 49). Judging from our results, it seems likely that the degree of hydrophobicity does not play a major role neither in the formation, nor the enzymatic removal, of the plaque film. Under comparable enzyme activities, a tendency was observed for more complete deposit removal from the hydrophilic glass surfaces used in the study of the influence of surface energy on *in vitro* plaque, than from those used in the study of the influence of enzymatic activity. However, this may be due to the different cleaning procedures employed for the surfaces.

In conclusion, the ability of the enzyme mixture Krillase to modify the salivary pellicle and remove adherent plaque bacteria, as well as to inhibit binding of oral microbes to saliva-coated surfaces without affecting the microbial viability, was demonstrated. Krillase has a broad spectrum of substrates, and it may constitute a potentially interesting agent for chemical plaque control. It will be a challenge to develop Krillase formulations suitable for clinical applications and further to investigate the interactions between Krillase and excipients such as surfactants and other proteins.

Acknowledgements Go to: Choose Go

We thank Dr. Stefan Welin-Klintström, Linköping University, for providing the oxidised silicon wafers and Dr. Kristian Hellgren for fruitful discussions. This work was financed by Arcimboldo AB, Sweden, Institute for Research and Competence Holding AB (IRECO), Sweden, and the Swedish Medical Research Council grant number 05712.

References Go to: Choose Go

1. Rosan B & Lamont RJ. Dental plaque formation. *Microbes Infect* 2000; 2: 1599-

1607.
MEDLINE

2. Nyvad B. Microbial colonization of tooth surfaces. *APMIS* 1993; **101** (Suppl 32): 1-45.
MEDLINE

3. Addy M, Slayne MA, Wade WG. The formation and control of dental plaque - an overview. *J Appl Bacteriol* 1992; **73**: 269-278.
MEDLINE

4. Marsh PD & Bradshaw DJ. Dental plaque as a biofilm. *J Ind Microbiol* 1995; **15**: 169-175.
MEDLINE

5. Jensen JL, Lamkin MS, Oppenheim FG. Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions. *J Dent Res* 1992; **71**: 1569-1576.
MEDLINE

6. Tabak LA, Levine MJ, Jain NK, Bryan AR, Cohen RE, Monte LD, Zawacki S, Nancollas GH, Slomiany A, Slomiany BL. Adsorption of human salivary mucins to hydroxyapatite. *Arch Oral Biol* 1985; **30**: 423-427.
MEDLINE

7. Glantz P-O & Baier RE. Recent studies on nonspecific aspects of intraoral adhesion. *J Adhesion* 1986; **20**: 227-244.

8. Carlén A, Bratt P, Stenudd C, Olsson J, Strömberg N. Agglutinin and acidic proline-rich protein receptor patterns may modulate bacterial adherence and colonization on tooth surfaces. *J Dent Res* 1998; **77**: 81-90.
MEDLINE

9. Gibbons RJ. Bacterial adhesion to oral tissues: a model for infectious diseases. *J Dent Res* 1989; **68**: 750-760.
MEDLINE

10. Gibbons RJ. Role of adhesion in microbial colonization of host tissues: a contribution of oral microbiology. *J Dent Res* 1996; **75**: 866-870.
MEDLINE

11. Gibbons RJ & Hay DI. Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces. *Infect Immun* 1988; **56**: 439-445.
MEDLINE

12. Mombelli A. Antibiotics in periodontal therapy. In: Lindhe J, Karring T, Lang NP, eds. Clinical periodontology and implant dentistry 3rd edition. Copenhagen: Munksgaard, 1998; 488-507.

13. Steinberg D & Rothman M. Antibacterial effect of chlorhexidine on bacteria adsorbed onto experimental dental plaque. *Diagn Microbiol Infect Dis* 1996; **26**: 109-115.
MEDLINE

14. Simonsson T, Arnebrant T, Petersson L. The effect of delmopinol on salivary pellicles, the wettability of tooth surfaces *in vivo* and bacterial cell surfaces *in vitro*. *Biofouling* 1991; **3**: 251-260.

15. Rundegren J, Simonsson T, Petersson L, Hansson E. Effect of delmopinol on the cohesion of glucan-containing plaque formed by *Streptococcus mutans* in a flow cell system. *J Dent Res* 1992; **71**: 1792-1796.
MEDLINE

16. Waaler SM, Rölla G, Skjörland KK, Ögaard B. Effects of oral rinsing with

triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. *Scand J Dent Res* 1993; **101**: 192-195.
MEDLINE

17. Listgarten MA, Lindhe J, Hellden L. Effect of tetracycline and/or scaling on human periodontal disease. Clinical, microbiological, and histological observations. *J Clin Periodontol* 1978; **5**: 246-271.
MEDLINE

18. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. *J Dent Res* 1992; **71**: 1431-1438.
MEDLINE

19. Moran J, Addy M, Newcombe R. Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpaste on the development of plaque and gingivitis. *J Clin Periodontol* 1989; **16**: 295-299.
MEDLINE

20. Hull PS. Chemical inhibition of plaque. *J Clin Periodontol* 1980; **7**: 431-442.
MEDLINE

21. Robinson RJ, Stoller NH, Vilardi M, Cohen DW. Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults. *Community Dent Oral Epidemiol* 1975; **3**: 271-275.
MEDLINE

22. Budtz-Jørgensen E. Prevention of denture plaque formation by an enzyme denture cleanser. *J Biol Buccale* 1977; **5**: 239-244.
MEDLINE

23. Budtz-Jørgensen E. A 3-months' study of enzymes as denture cleansers. *J Oral Rehabil* 1978; **5**: 35-39.
MEDLINE

24. Budtz-Jørgensen E, Kelstrup J, Poulsen S. Reduction of formation of denture plaque by a protease (Alcalase). *Acta Odontol Scand* 1983; **41**: 93-98.
MEDLINE

25. Kelstrup J, Funder-Nielsen TD, MØller EN. Enzymatic reduction of the colonization of *Streptococcus mutans* in human dental plaque. *Acta Odontol Scand* 1973; **31**: 249-253.
MEDLINE

26. Kelstrup J, Holm-Pedersen P, Poulsen S. Reduction of the formation of dental plaque and gingivitis in humans by crude mutanase. *Scand J Dent Res* 1978; **86**: 93-102.
MEDLINE

27. Ödman PA. The effectiveness of enzyme-containing denture cleanser. *Quintessence Int* 1992; **23**: 187-190.
MEDLINE

28. Anheller J-E, Hellgren L, Karlstam B, Vincent J. Biochemical and biological profile of a new enzyme preparation from Antarctic krill (*E. superba*) suitable for debridement of ulcerative lesions. *Arch Dermatol Res* 1989; **281**: 105-110.
MEDLINE

29. Hellgren L, Karlstam B, Mohr V, Vincent J. Peptide hydrolases from antarctic krill - an important new tool with promising medical potential. In: Margesin R, Schinner F, eds. *Biotechnological applications of cold-adapted organisms* . Berlin: Springer-Verlag, 1999; 63-74.

30. Azzam RMA & Bashara NM. *Ellipsometry and polarized light* . Amsterdam:

North-Holland, 1977.

31. Bucht A & Karlstam B. Isolation and immunological characterization of three highly purified serine proteinases from Antarctic krill (*Euphausia superba*). *Polar Biol* 1991; **11**: 495-500.
32. Karlstam B. Crossed immunoelectrophoretic analysis of proteins from Antarctic krill (*Euphausia superba*) with special reference to serine proteinases. *Polar Biol* 1991; **11**: 489-493.
33. Karlstam B, Johansson B, Brynö C. Identification of proteolytic isozymes from Antarctic krill (*Euphausia superba*) in an enzymatic debrider. *Comp Biochem Physiol* 1991; **100B**: 817-820.
34. Osnes KK. *Peptide hydrolases of Antarctic krill*, Euphausia superba. Doctoral thesis, Norwegian Institute of Technology, Trondheim, Norway, 1985.
35. Elwing H, Welin S, Askendal A, Nilsson U, Lundström I. A wettability gradient method for studies of macromolecular interactions at the liquid/solid interface. *J Colloid Interface Sci* 1987; **119**: 203-210.
36. Wannerberger K, Welin-Klintström S, Arnebrant T. Activity and adsorption of lipase from *Humicola lanuginosa* on surfaces with different wettabilities. *Langmuir* 1997; **13**: 784-790.

37. Wahlgren M & Arnebrant T. Adsorption of β-lactoglobulin onto silica, methylated silica, and polysulfone. *J Colloid Interface Sci* 1990; **136**: 259-265.
38. Landgren M & Jönsson B. Determination of the optical properties of Si/SiO_2 surfaces by means of ellipsometry, using different ambient media. *J Phys Chem* 1993; **97**: 1656-1660.
39. Dawes C. Rhythms in salivary flow rate and composition. *Int J Chronobiol* 1974; **2**: 253-279.

40. De Feijter JA, Benjamins J, Veer FA. Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air-water interface. *Biopolymers* 1978; **17**: 1759-1772.
41. Cuypers PA, Corsel JW, Janssen MP, Kop JMM, Hermens WT, Hemker HC. The adsorption of prothrombin to phosphatitylserine multilayers quantitated by ellipsometry. *J Biol Chem* 1983; **258**: 2426-2431.

42. Vassilakos N, Arnebrant T, Glantz P-O. Adsorption of whole saliva onto hydrophilic and hydrophobic solid surfaces. The influence of concentration, ionic strength and pH. *Scand J Dent Res* 1992; **100**: 346-353.

43. Lindh L, Arnebrant T, Isberg P-E, Glantz P-O. Concentration dependence of adsorption from human whole resting saliva at solid/liquid interfaces: an ellipsometric study. *Biofouling* 1999; **14**: 189-196.
44. Kolenbrander PE & London J. Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 1993; **175**: 3247-3252.

45. Mosbach K. Enzymes and general biology: special reference to enzyme synthesis, purification, and reactions on matrixes. *J Dent Res* 1972; **51**: 217-223.

46. Quirynen M & Bollen CML. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol* 1995; **22**: 1-14.


47. Vassilakos N, Glantz P-O, Arnebrant T. Reflectometry: a new method for quantitative determinations of intraoral film formation. *Scand J Dent Res* 1993; **101**: 339-343.
MEDLINE

48. Glantz P-O. *On wettability and adhesiveness*. Doctoral thesis, Lund University, Lund, Sweden, 1969.

49. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Arends J, Darius PL, van Steenberghe D. The influence of surface-free energy on planimetric plaque growth in man. *J Dent Res* 1989; **68**: 796-799.
MEDLINE

In the United States Patent and Trademark Office

In re: deFaire	BOARD OF PATENT APPEALS AND INTERFERENCES
Serial No.: 09/549,642	
Filing Date: April 14, 2000	
For: REMOVING DENTAL PLAQUE WITH KRILL ENZYMES	PATENT APPEAL
Docket No.: 314572-101F	
Art Unit: 1642	
Examiner: Brumback, B.	

APPELLANT'S APPEAL BRIEF

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TABLE OF CONTENTS

(1) Real Party in Interest

The inventors have assigned their interest to Phairson Medical Inc. is the real party in interest.

(2) Related Appeals and Interferences

On information and belief, there are no other appeals or interferences that will directly affect or have a bearing on the Board's decision in this Appeal.

(3) Status of the Claims

Claims 142-144 are in the application. All other claims have been cancelled. All of the pending claims are subject to one or more rejections under 35 U.S.C. §§102(b), 103(a) and 112. Hence, claims 142 through 144 are appealed.

(4) Status of Amendments Filed After Final

There are no amendments after final. The pending claims are attached as Appendix A.

(5) Summary of Invention

The invention rests on the observation that the hydrolases extracted from Krill are very effective in removing plaque. Such an extract was known to be useful as a cleaning agent – but plenty of enzymes used in cleaning are ineffective to remove dental plaque.

(6) Issues

The issues are:

(i) *Issue 1:* Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?

(ii) *Issue 2*: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

(iii) *Issue 3*: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

(iv) *Issue 4*: Are the phrases "wherein the dental plaque is visually observable" in claim 143 and "until dental plaque is not longer visually observable" in claim 144 supported in the specification?

(7) **Grouping of claims**

The three claims in this application each raise separate issues with respect to one or more of the issues, and shall therefor be argued separately. Accordingly, there is no grouping of the claims.

(8) **Argument**

(i) *Issue 1*: **Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?**

The rejection under 35 U.S.C. §102(b) is premised on principles of inherent anticipation, since an electronic word search of the cited document confirms that not one word relates to any type of plaque. As discussed in M.P.E.P. §2112, such a rejection requires that the element not expressly set forth in the reference necessarily flows from the disclosures of the reference. This requirement is not met here.

The 'Hellgren patent is – in its core discussions – about cleaning wounds and objects such as textiles. One word in the specification mentions that teeth can be cleaned (at 1:31[1]). There is no concrete exemplification from which to infer what type of teeth are to be treated, or even that teeth *in situ* are to be treated. Teeth cleaning occurs often without plaque being effectively removed, or without plaque being present. Thus, treating to effectively remove plaque, as claimed, does not necessarily follow from the brief mention of teeth cleaning in the Hellgren patent.

The most recent Office Action asserts that contacting teeth with the enzymes recited necessarily removes dental plaque. In a sense this is true: If you picked the right amount and

---

[1] Column 1, line 31.

application of the enzymes, if you picked teeth that had plaque, you would effectively remove dental plaque as illustrated in the Examples of Appellant's specification. However, the prior art disclosure must teach one what the effective conditions are and what to treat: the cited Hellgren patent does not. Thus, this would only necessarily follow if you made many choices not taught by Hellgren and not necessarily following from Hellgren. Thus, Appellant would submit that this compilation of untaught selection is not what is required to satisfy the "necessarily" standard. Hellgren teaches nothing at all about how much enzyme composition to apply, and does not even teach applying the treatment to teeth that have plaque. So for anticipation, Hellgren is clearly lacking that which would indicate that its methodology (of which, for this dental application, there is none) would necessarily achieve removal of plaque. The rejection is simply not supported by the cited document and should be withdrawn.

The rejection has been extended to claim 143, but nowhere do the Office Actions describe how the cited art teaches selecting subjects where "the dental plaque is visually observable." As the complete disclosure of the cited document is described above, it should be clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

The rejection has been further extended to claim 144, and again the Office Actions do not describe how the cited art teaches that the contacting "is conducted or repeated until dental plaque is not longer visually observable." Since the complete disclosure of the cited document is described above, again it is clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

(ii) *Issue 2*: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

Applicants respectfully submit that the disclosure in Hellgren that krill enzymes can be used to debride wounds or clean textiles does not render the claims obvious. While the results obtained in the debriding and textile cleaning context may be strong, nonetheless enzymes have been used in cleaning textiles for quite some time, and similarly there is a long history of use in

wound debridement. Thus, effectiveness in a different, highly complex environment, namely dental plaque, would not have been apparent to one of ordinary skill.

The complexity of dental plaque is illustrated by the following discussion in US Patent 6,159,447 (a patent which relates to treating plaque with an anchored enzyme):

> Plaque is a heterogeneous mixture of bacterial aggregations embedded in a sticky matrix. While bacterial composition of plaque ranges from 50 to 70 percent, the matrix is derived from dead cells, salivary glycoproteins and serum proteins that are laid on a polysaccharide backbone. The bacteria synthesize the polysaccharides for the plaque backbone as a step in their own colonization process. In addition to the viable bacteria and the matrix, plaque also contains food debris, small numbers of epithelial cells, white blood cells and various other components which are derived from the host and the host's activities.
>
> The formation and development or proliferation of plaque occurs in two stages. The first step may require a base layer of salivary glycoproteins on the tooth's surface as well as on the soft tissue in the oral cavity. This base organic layer, derived from saliva, is adsorbed onto the surface and forms an acquired pellicle. This insoluble acquired pellicle serves as the foundation for supragingival plaque. The second step is the bacterial colonization by "pioneering" bacteria of the acquired pellicle. Once the bacteria have attached to the surface of a structure, they aggregate, develop colonies and plague begins to form.
>
> There are well over 100 different bacterial species in various dental plaques. This variation in the types of bacteria is influenced by diet, salivary components and bacterial interactions, to name a few. The location of the plaque in the oral cavity, the time of the day, age of the patient and the status of the general oral hygiene of the patient all contribute to the implications and consequences of dental plaque and periodontal disease. Consequently, it is not surprising that plaque is a heterogeneous collection of bacterial communities attached to the tooth providing a vast array of biochemical and physiological consequences. Two major pathological conditions as consequences are periodontal disease and dental caries.

> Enzymes as therapeutic agents present unique possibilities. However, some of the early oral pathology research using enzymes was based on the assumption that they would be bactericidal to colonies of organisms found in plaque and therefore would act as "disinfectants". This approach, however, was not fruitful. Recently, it was shown that treatment of buccal epithelial cells with protease altered bacterial adhesion; however, this treatment also distorted the ratios of various bacterial populations. More promising results were obtained when the focus was shifted from bactericidal action to altering plaque formation. These latter results were seen in vitro and in vivo as well as in animal models and humans in clinical trials. However, these approaches also fell short of desired therapeutic effectiveness most likely because the required time for an effective action exceeded the retention time of the enzyme in the oral cavity. In short, salivary flow, other fluid and food movement and normal mechanical agitation in the oral cavity reduced the retention time of the enzyme(s). These factors shortened the residence time of the enzymes, resulting in less than desirable clinical efficacy.

Thus, the above discussion indicates not just that dental plaque is complex, but that other enzymes have apparently failed in removing dental plaque. Further, the publication abstracts attached as Exhibits A-C indicate further that enzyme cleaners can fail to reduce plaque. Accordingly, the cited Hellgren document describes using the enzyme to clean teeth – it does not motivate experimenting to find conditions effective to remove plaque.

Applicant submits that the fact that the type of enzyme mixture recited in the claims would be effective in removing plaque, as illustrated in Examples 33 and 34, and further illustrated in Exhibit D, was obvious only after the Appellant presented the result. Thus, the rejection represents an inappropriate hindsight reconstruction of the invention. Since it would not be apparent that teeth cleaning with the enzymes would produce plaque removal, one would not, absent hindsight, select subjects with visibly apparent plaque so as to satisfy claim 143. It would be further less apparent to continue or repeat the treatment until "dental plaque is not longer visually observable," as required by claim 144.

Accordingly, Appellant submits that this rejection should be withdrawn.

(iii) *Issue 3*: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

Karistam is cited for substantially the same disclosure as is found in Hellgren, but perhaps further including the disclosure that krill enzymes include a hyaluronidase activity that degrades a glycoaminoglycan. Ratcliff is asserted in an Office Action to teach:

> that dental plaque is a complex extracellular matrix containing glucosaminoglycans, chondroitin sulfates, glycoproteins, and proteins. Ratcliff further teaches that degradation of these compounds retards plaque growth.

The March 29, 2001 Office Action cites for these propositions the following text italicized text from Ratcliff:

> Bacterial agglutinigation includes the conversion of sucrose to glucans and fructans by enzymes known as glycosyltransferases. These enzymes are of bacterial origin. The plaque maas [sic] becomes a complex extra cellular (of microorganisms) matrix containing sulphated glucosamineglycans, proteoglycans, glycoproteins, sugar, proteins and lipids which aid in the process of bacterial agglutination. These compounds include the presence of sulphur and become unstable in the presence of high oxygen compounds. The oxygen splits the sulphide bonds to form sulphates or SO_2.
>
> Clinical observations by the inventor have led to the conclusion that **all of these biochemical compounds are attacked to a greater or lesser extent by stabilized chlorine dioxide**. Since these compounds may be used as nutrients for bacteria, the reduction of the compounds will inhibit bacterial growth. More specifically, the stabilized chlorine dioxide oxidizes carbohydrates, chondroitin sulphates, glucosaminglycans, glycoproteins, proteins and lipids. Since these compounds arise as bacterial by products and debris from dead and dying cells, are of salivary origin and are the mechanism of agglutination of the plaque mass, their degradation/oxidation retards plaque growth.

Ratcliff at 3:62 – 4:22 (citation omitted).

Applicant would respectfully submit that Ratcliff indicates that **stabilized chlorine dioxide** attacks the complex mixture of components of plaque. This teaching has no bearing on whether or not Hellgren's composition would be effective. In fact, it teaches that what the Merck Index describes as a "strongly oxidizing" gas that "reacts violently with organic compounds" is required. The rejection is simply in error.

Accordingly, Applicant respectfully submits that the rejection is in error, and should be withdrawn.

(iv) *Issue 4*: Is the phrase "wherein the dental plaque is visually observable" in claim 143 supported in the specification?

Claims 143 and 144 stand rejected under 35 U.S.C. §112, first paragraph, based on an assertion of lack of support in the specification. This support is clear from the two relevant working examples.

The two relevant examples in the specification read:

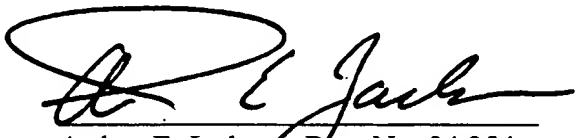
> The krill poly-enzyme preparation was used to remove dental plaque in beagles. Before use, ampoules of the poly-enzyme preparation (described in Example 1A) were reconstituted in 5 ml of saline to a final concentration of 5 Casein-Units/ml. The content from a freshly prepared ampoule was carefully painted over teeth and gingiva. The tongue was fixated for 2 minutes and food and beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily until all plaque was completely decomposed. The dogs were ***inspected*** for status of plaque, saliva secretion and adverse reactions once daily. Eight beagles with abnormal plaque formation due to special feeding and housing were included in this study. After 4 days ***all signs of plaque*** were gone and the study was terminated. No adverse reactions could be observed.
>
> The krill poly-enzyme preparation was used to remove dental plaque in humans. One ampoule of poly-enzyme preparation of Example 1A was reconstituted, in 5 ml of saline to a final concentration of 5 Casein-Units/ml, before each treatment and used to rinse the patient's mouth cavity for 5 minutes. Food and

beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily and the patients were ***inspected once daily for plaque***, saliva secretion, dryness, and adverse reactions. The patients were not allowed to brush their teeth during the study period. The treatment was continued until ***all signs of plaque*** were gone, but not for longer than 7 days. Two hours after the first treatment all patients experienced a soft and smooth sense over their teeth but ***visual inspection showed remnants of plaque***. Two hours after the third treatment, ***all signs of plaque were gone*** and treatments were terminated. No adverse reactions were observed.

From the above, it should be quite clear that the subjects had visually apparent signs of plaque, and that appropriate treatment can be conducted to remove such visual signs. Accordingly, the specification supports the questioned recitations. Therefore the rejections should be withdrawn.

CONCLUSION

For the foregoing reasons, Appellant respectfully requests that the rejections under 35 U.S.C. §§ 102, 103 and 112 with respect to all of the pending claims be reversed and the pending claims in the application allowed.



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Dated: August 12, 2002

Appendix:

Claims on Appeal.

Exhibits:

A: Abstract of J. Clin. Periodontol 7:431-42, 1980.
B: Abstract of J. Clin. Periodontol 16:295-9, 1989.
C: Abstract of Community Dent. Oral Epidemiol. 3:271-5, 1975.
D: Berg et al., "Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control I," European J. Oral Sciences 109:316, 2001.

APPENDIX A - COPY OF CLAIMS ON APPEAL

142. A method of removing dental plaque in an animal subject comprising: contacting the dental plaque with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

143. A method of removing dental plaque comprising:
contacting the dental plaque, wherein the dental plaque is visually observable, with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

144. The method of claim 143, wherein the contacting is conducted or repeated until dental plaque is not longer visually observable.

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1: J Clin Periodontol 1980 Dec;7(6):431-42 Related Articles, Books, Link

Chemical inhibition of plaque.

Hull PS.

Attempts to control plaque by chemical means using enzymes, antibiotics an antiseptics are reviewed. Enzymes such as mucinase, dehydrated pancreas, enzymes of fungal origin, dextranase and mutanase showed limited clinical success despite promising in vitro and animal studies. Side effects from the of enzymes were observed. Many antibiotics have been used in attempts to control plaque and several have been successful. However, problems exist from the long-term use of such drugs which precludes their routine use as agents for controlling plaque. The biguanide chlorhexidine is the most widel used and investigated method of chemical plaque control. Many studies hav been demonstrated that it will successfully control plaque. No toxic side effects have been reported from its long-term use but local side effects such staining of the teeth do occur. The quaternary ammonium compounds have a present no advantages over the biguanides and require more frequent usage t achieve the same degree of plaque control as chlorhexidine.

Publication Types:
- Review

PMID: 7012186 [PubMed - indexed for MEDLINE]

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B

1: J Clin Periodontol 1989 May;16(5):295-9 Related Articles, Books, Link

Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpas on the development of plaque and gingivitis.

Moran J, Addy M, Newcombe R.

Department of Periodontology, Dental School, University of Wales College Medicine, Heath Park, Cardiff, UK.

Many toothpastes have been formulated over recent years to contain antimicrobial compounds with the aim of preventing or reducing plaque, calculus, gingival inflammation or dental caries. For many, if not all of these toothpastes, it has yet to be proven whether they are significantly better at reducing plaque and gingivitis than conventional toothpastes, for which no such therapeutic effects have been claimed. This 12-day, incomplete block designed, cross-over study compared the development of plaque and gingivi following rinsing with toothpaste slurries containing the following active ingredients: (1) hexetidine/zinc citrate, (2) 0.2% triclosan, (3) amyloglucosidase/glucose oxidase, (4) sodium fluoride/sodium monofluorophosphate (NaF, MFP). By the 8th day of the study, a significant difference in gingival crevicular fluid (GCF) and GI was found between the groups. By day 12, however, no significant difference in plaque index and gingival inflammation was found between the 4 toothpastes, although plaqu area was significantly reduced with the hexetidine/zinc citrate paste when compared to the conventional fluoride paste. It was concluded that the active ingredients added to the toothpastes evaluated in this study provided little or no more additional benefit to oral hygiene and gingival health than could be achieved with a conventional fluoride toothpaste.

Publication Types:
- Clinical Trial
- Controlled Clinical Trial

PMID: 2498397 [PubMed - indexed for MEDLINE]

1: Community Dent Oral Epidemiol 1975 Nov;3 (6):271-5

Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults.

Robinson RJ, Stoller NH, Vilardi M, Cohen DW.

One hundred and thirty-one young adults were used in a controlled study to determine whether a proteolytic mouthwash produced from B. subtilis could help remove existing plaque, decrease the rate of plaque accumulation or reduce the clinical signs of gingivitis. These subjects were stratified by mea of the Gingival Index and the Shaver-Schiff Plaque Index into severe or mil groups. These groups were further divided in a random fashion into treatme and placebo groups. The treatment group rinsed with a proteolytic mouthwa consisting mainly of neutral and alkaline proteases and amylase. A placebo mouthwash was used by the control group. The results of this investigation indicate that there is no statistically significant reduction either in existing plaque or in the rate of plaque accumulation when this proteolytic enzyme mouthwash is used. Furthermore, in the treatment group the degree of gingivitis as measured by the GI was not reduced.

Publication Types:
- Clinical Trial
- Randomized Controlled Trial

PMID: 1104247 [PubMed - indexed for MEDLINE]

Full Article

View/Print PDF article (493K)

Eur pean Journal Of Oral Sciences
Volume 109 Issue 5 Page 316 - October 2001

Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control

I. Cecilia Hahn Berg [1], Sotirios Kalfas [2,3], Martin Malmsten [1,4], Thomas Arnebrant [1,5]

This paper deals with enzymatic removal of dental plaque, *in vitro* as well as *in vivo*, using proteases from the Antarctic krill shrimp (*Euphausia superba*), referred to as Krillase®. Krillase exhibits both endo- and exopeptidase activity but has no microbicidal effect. In model systems with pure cultures of oral microorganisms, Krillase demonstrated inhibition of microbial adhesion to saliva-coated hydroxyapatite. Furthermore, a protocol for the growth of reproducible *in vitro* plaque films has been developed, and effects of Krillase on the plaque film were investigated by means of scanning electron microscopy (SEM). The results showed that Krillase efficiently released microorganisms from plaque *in vitro*, the effect being dependent on the enzymatic activity. The surface energy of the substratum had a minor influence on the formation and removal of plaque *in vitro*. Ellipsometric studies on the formation and enzymatic removal of a salivary pellicle indicated that the enzymatic effect on plaque may partly depend on degradation of the salivary pellicle. Krillase was also able to remove plaque accumulated on dentures *in vivo*. Our results demonstrate the potential of Krillase for plaque control, and that these enzymes are worthy of further investigations including clinical studies and work to find a suitable vehicle.

Accumulation of microbial plaque on tooth surfaces is the cause of caries and periodontal diseases, the most prevalent oral disorders that affect industrialised societies and account for the considerable research interest paid to the subject during the last decades. Plaque development proceeds through various phases (1-4). Initially, salivary proteins such as acidic proline-rich proteins, statherin, histatins, amylase and mucins adsorb rapidly to the solid surface and form a salivary pellicle (5, 6). The pioneer bacterial colonisers adhere to the pellicle within the next couple of hours, employing various strategies ranging from non-specific physicochemical attachment (7) to recognition of specific receptor sites at the salivary proteins and using cell surface fimbriae (8-11). Other species co-adhere with the primary colonisers, and a complex microbial community is then developed through growth and stratification of the bacteria embedded in a matrix of polymers of bacterial and salivary origin.

Control of plaque accumulation can be achieved by mechanical methods such as brushing and flossing, and by use of chemical substances such as surfactants and antibiotics (12-17). If done properly, mechanical means will give adequate results, but there are also occasions when chemical plaque control is advantageous as a complement in order to counteract plaque formation. The main disadvantage of the latter methods is that the chemicals may selectively inhibit different populations of

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bacteria, resulting in disturbances of the balance in the oral ecosystem. Emergence of resistant bacterial clones upon frequent use of antimicrobial substances is another factor limiting extensive use of some anti-plaque agents.

Enzymes with a broad spectrum of specificity for bacterial surface structures and polymers in the extracellular matrix of dental plaque have also been tried for chemical control of plaque (18). These agents usually lack the above disadvantages and have comparably low antimicrobial profile. The efficacy of enzyme formulations in removing dental or denture plaque varied in clinical studies (19-27). The substrate specificity of the enzymes tested and the administration methods employed have certainly influenced the results in the different studies.

A natural mixture of digestive proteases (referred to as Krillase®) extracted from the Antarctic krill shrimp *Euphausia superba* was earlier characterized and tested for debridement of ulcerative lesions (28, 29). Krillase consists of endo- and exopeptidases able to degrade complex proteinaceous substrates more efficiently than single-enzyme preparations. In addition, the enzymes in Krillase have naturally been selected to co-exist, which makes the autolysis rate of Krillase low. These specific properties of Krillase provided the incentive to conduct this study to examine the effect of Krillase on bacterial viability and adhesion to saliva-coated surfaces as well as the ability of Krillase to counteract formation of salivary pellicle and microbial plaque.

In order to study events at the molecular level, ellipsometry was employed. Based on the measurement of polarisation changes of light upon reflection, ellipsometry enables monitoring of adsorption/desorption processes *in situ* with a time resolution in the order of seconds (30).

Material and methods

Go to: Choose Go

Chemicals

Tris (tris[hydroxymethyl]aminomethane, ≥99% purity) and bovine serum albumin (≥96% purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). (3, 3-dimethylbutyl)dimethylchlorosilane (97% purity) was from ABCR (Karlsruhe, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). The water used in the *in vitro* plaque and ellipsometry experiments was treated by a Milli-Q Plus unit (Millipore, Bedford, MA, USA) including ion exchange, active carbon adsorption and reverse osmosis before the final 0.22- μ m filtration step, yielding ultrapure reagent-grade water of resistivity 18.2 MΩcm at 25°C. In all the other experiments, de-ionised water was used. Ethanol (99.7% pure) was from Primalco (Helsinki, Finland) and Krillase was obtained from BioPhausia (Uppsala, Sweden) (batch numbers B038912, 610008, 604040 and 710017). The lyophilised preparation was reconstituted in water (2 ml/vial) and further diluted with 0.1 M Tris-HCl buffer, pH7.5, to obtain the appropriate concentration (total proteolytic activity as determined by the supplier and expressed in units per ml solution, U/ml). The characteristics of the enzyme mixture have been reported elsewhere (28, 31-34).

Effect on bacterial viability

Streptococcus mutans NCTC 10449, *Streptococcus sanguis* ATCC 10556, *Actinomyces naeslundii* ATCC 12104, and *Candida albicans* CHR (own isolate) were grown at 37°C for 24 h in the presence of 5% CO_2 on *Brucella* agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% lysed blood, 0.05 mg/ml hemin and 0.01 mg/ml vitamin K. Cells harvested with a sterile cotton swab were suspended in sterile A-buffer (1 mM sodium phosphate buffer containing 50 mM KCl, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH7.0) and the optical density (at 600 nm) of the suspension was adjusted to 0.5.

Cell suspension (final density 10^6-10^8 cells/ml) was mixed with Krillase solution (final activity 1 U/ml) and A-buffer to a final volume of 1 ml. In the controls, the

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Image Previews

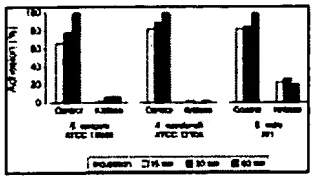

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Fig.1 Relative (%) number of bacteria adherent to saliva-coated hydroxyapatite beads at different inc...

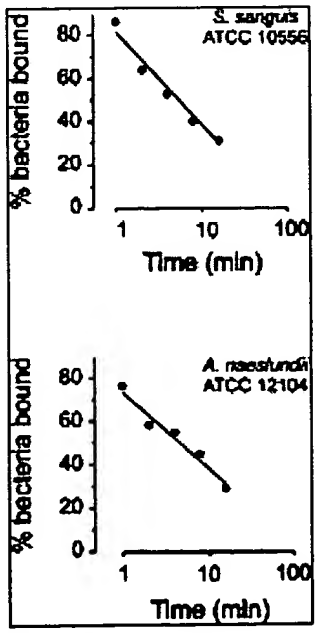

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Fig.2 Kinetics for Krillase-dependent (activity 0.5 U/ml) detachment of *S. sanguis* and *A. naeslundii*... ...

Krillase solution was replaced by A-buffer. The assays were run aerobically at 37° C. Samples were taken from the mixtures after 15, 45 and 75min of incubation and diluted serially in cold 0.1 M potassium phosphate buffer (pH6.8). From appropriate dilutions, 0.1-ml aliquots were inoculated on *Brucella* blood agar plates and incubated for 48h at 37°C in 5% CO_2. The number of viable bacteria in the samples was determined from the number of colonies grown on the plates and is expressed in colony-forming units (CFU) per ml.

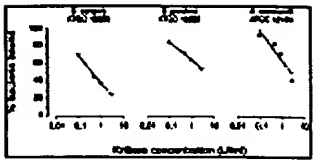

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Fig.3 Effect of enzymatic activity on the Krillase-dependent detachment of *S. sanguis*, *S. gordonii* a...

Effect on bacterial adhesion to saliva-coated hydroxyapatite

Streptococcus mitis St1 (own isolate), *S. sanguis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, and *A. naeslundii* ATCC 12104 were grown in Trypticase soy broth (Becton Dickinson) supplemented with 0.5% yeast extract (Difco, Detroit, MI, USA) and 5μCi/ml [^{3}H]thymidine (Amersham, Little Chalfont, Bucks., England) as described above. Cells were harvested by centrifugation (6000× *g* at 6°C for 10 min) and washed twice with 5ml A-buffer before suspended in A-buffer to yield an optical density of 2.0 at 600nm.

Porous hydroxyapatite beads (lot number 011093-01; Clarkson Chromatography Products, South Williamsport, PA, USA) were coated with paraffin-stimulated whole saliva (SHA beads). The saliva was freshly collected on ice and clarified by centrifugation (16,000× *g*, 6°C, 10min) before used. Alkali-washed hydroxyapatite beads were rehydrated with A-buffer overnight before incubation with saliva (40mg beads/ml saliva) at 6°C under rocking for 1h. The saliva was removed, and the beads were washed with 2×2ml of A-buffer followed by a 30-min incubation with A-buffer containing 0.5% bovine serum albumin. Finally, the beads were washed with 3×3ml of A-buffer.

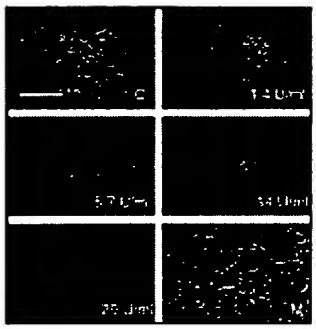

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Fig.4 SEM images of *in vitro* plaque on glass slides incubated for 15min in Tris-HCl buffer (control ...

Cell suspension (final density about 10^8 cells/ml) was mixed with 40mg SHA beads and Krillase solution (final activity 1U/ml). Sterile A-buffer was added to a total volume of 1ml. The enzyme solution was replaced by pure A-buffer in the controls. The mixtures were incubated aerobically at 37°C under continuous rocking. After 15, 30, and 60min of incubation, the supernating suspension was removed, and the beads were washed with 3×1ml of A-buffer containing 0.1% formalin in order to stop the enzymatic activity and remove non-adherent cells. The beads were thereafter mixed with 10ml of scintillation cocktail (Biosafe; Beckman, Fullerton, CA, USA) and the radioactivity from bacteria adherent to the beads was counted in a β-counter (Rackbeta; LKB Wallac, Turku, Finland). The number of bacteria adherent to the beads was calculated from the radioactivity of the samples. Relative adhesion is given as a percent of the maximum value obtained in the control mixture at 60min.

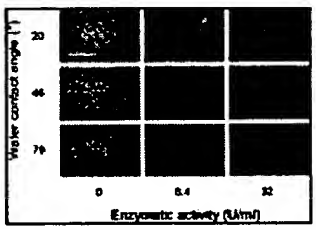

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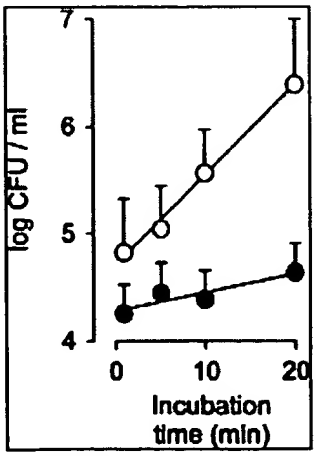
Fig.5 SEM images of *in vitro* plaque on glass slides with various surface energy, exposed to either T...

In order to examine the effect of incubation time and enzymatic activity on the anti-adhesive properties of Krillase, cell suspension (final density about 10^8 cells/ml) was mixed with SHA beads (1ml suspension to 200mg beads) and incubated aerobically at 37°C for 45min under continuous rocking. The cell suspension was removed, and the beads were washed twice with 2ml of A-buffer in order to remove non-adherent cells. Krillase solution (0.5ml, 0.5U/ml) was added, and the mixture was incubated at room temperature for defined periods of time. In separate experiments, Krillase at various activities was added to the beads, and the mixture was incubated for 5min. The assay was terminated by the addition of 1ml 2% formalin in A-buffer. The suspension was removed, and the beads were washed twice with 2ml A-buffer in order to remove non-adherent cells. The beads were thereafter mixed with 0.5ml 1M KOH and incubated for 10min before the solution was neutralised with 1M HCl. The beads and the liquid phase were mixed with 10 ml of scintillation cocktail, and the radioactivity was counted as described above. Relative adhesion is given as percent of the maximum value obtained in the control mixtures (no enzyme added or 0min incubation time).



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Effect on plaque formed *in vitro*

In order to obtain *in vitro* plaque, saliva samples obtained after gustatory stimulation with a sugar lump were poured into polystyrene Petri dishes (about 8ml

saliva per dish) containing glass slides (1×1 cm^2 each) that had been rinsed with water and ethanol and air-dried. The dishes were incubated at 37°C for 48h. The plaque formed on the slides was rinsed twice with water, and excess water was absorbed carefully from the slide edges. The slides were then immersed for 15min in dishes containing warm (37°C) solution of Krillase in 0.1M Tris-HCl buffer, pH 7.5, or buffer alone (control). The samples were rinsed twice with water and air-dried. The sample surfaces were coated with a thin layer (~90nm) of Au/Pd (2 ×180s, 40mA; Balzers Sputter Coater SCD 050) and then examined with a Philips scanning electron microscope (SEM 515) operating at 15kV.

In separate experiments, the influence of surface energy of the substratum on plaque formation and removal was studied. For this purpose, the glass slides used for plaque accumulation had been boiled (80°C) for 5min in a 1:1:5 (by volume) mixture of 25% NH_3, 30% H_2O_2 and water, followed by rinsing in water and a second boiling step as above. Finally, the slides were rinsed twice with both water and ethanol. This treatment yielded hydrophilic glass surfaces with a static water contact angle of 20°, as measured by goniometry. From the hydrophilic glass surfaces, hydrophobic surfaces of two different surface energies were prepared by means of liquid phase silanisation in either p-xylene or trichloroethylene (35, 36). After rinsing in ethanol and p-xylene or trichloroethylene, the slides were immersed in either 0.1% or 0.06% solutions of (3, 3-dimethylbutyl)dimethylchlorosilane in p-xylene or trichloroethylene for 15 or 90min, respectively. The slides were finally rinsed four times with p-xylene or trichloroethylene and four times in ethanol and then stored in ethanol. These procedures yielded surfaces with static water contact angles of 46° (p-xylene solution) and 79° (trichloroethylene solution).

Effect on plaque formed *in vivo*

Fifteen subjects wearing dentures and having a clinically healthy oral mucosa participated in the study. All participants obtained detailed information about the study. The participants visited the clinic on three occasions. On the first visit, the dentures were cleaned professionally with brush, water and detergent. The participants were thereafter instructed to wear their dentures as usual for the consecutive 2d and to refrain from any denture cleansing procedure, apart from rinsing with tap water after meals to remove food remnants. On the third day, the plaque-removing ability of Krillase was tested. Each denture was immersed in 500 ml of water and kept under gentle agitation on a magnetic stirrer for 1min in order to remove saliva and loosely attached food remnants. The denture was then immersed in 120ml of Krillase solution (1 U/ml) and incubated at 25°C for up to 20 min under gentle agitation by orbital rotation at 18rpm. At defined time points, samples were aspirated and diluted with 0.1M potassium phosphate buffer containing 0.5% NaCl (pH6.8). From appropriate dilutions, 0.05-ml aliquots were inoculated on *Brucella* blood agar plates. The plates were incubated aerobically at 36°C for 48h, and the total number of colonies was counted.

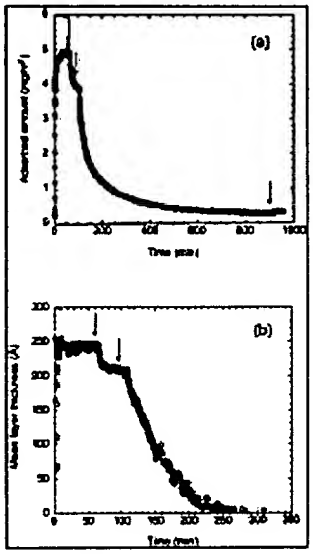
One week later, the subjects visited the clinic again having allowed plaque to accumulate on the dentures for 2d as outlined above. The assay for plaque removal was repeated, but on this occasion a heat-inactivated (20min in boiling water) Krillase solution was used to serve as a negative control.

Effect on pellicle formation

Silica slides (Si/SiO_2) used for ellipsometric experiments were oxidised and cleaned as described in detail elsewhere (37). The silica surfaces were hydrophilic with a static water contact angle of <10°. Prior to usage, the slides were stored in ethanol. The silica surfaces were plasma-cleaned for 5min immediately before the ellipsometric measurement. Cleaning was performed in low-pressure residual air using a radio frequency glow discharge unit (Harrick Plasma Cleaner PDC-3XG; Harrick Scientific, Ossining, NY, USA).

Adsorption of salivary proteins to silica surfaces was followed by null ellipsometry, using an automated Rudolph Research thin film ellipsometer (type 43603-200E; Rudolph Research Analytical, Flanders, NJ, USA), controlled by a personal computer and operated at an angle of incidence of 67.7°. A xenon lamp, filtered to

Fig.6 Release kinetics of microbes from dentures immersed in Krillase solution (1 U/ml, open symbols...



[Full Size]

Fig.7 Kinetics for salivary pellicle formation and enzyme-induced removal, as determined *in situ* by ...

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4015 Å, constituted the light source. The silica surface was mounted in a thermostated (25°C) quartz cuvette, agitated by a magnetic stirrer at about 90rpm. Each experiment was preceded by a four-zone calibration of the surface in two ambient media, air and buffer (10mM sodium phosphate buffer with 50mM NaCl, pH7.0), in order to determine the complex refractive index of the silicon and the refractive index and thickness of the oxide layer. A detailed description of the instrumental set-up and procedure can be found elsewhere (38). Unstimulated saliva, collected according to the procedure described by DAWES (39), was added to the buffer in the cuvette to a final concentration of 10% (v/v). The adsorption process was monitored continuously in one zone, taking into account the corrections for imperfections in optical components obtained from the four-zone calibration. In order to remove non-adsorbed salivary components, the pellicle formation was interrupted after 1h by rinsing with buffer for 35min. Krillase was then added to a final activity of 0.14U/ml, and following the degradation process (after 13h and 25min), the cuvette was finally rinsed for 45min. The flow rate used at rinsing was 1.6ml/min.

The thickness and mean refractive index of the adsorbed film are related to the output parameters of the ellipsometer, the ellipsometric angles Ψ and Δ. The adsorbed amount per unit area can be calculated (40), if the refractive index increment of the adsorbing molecules with concentration in solution (dn/dc) is known and assumed to be constant up to the concentration found in the adsorbed layer. The value of dn/dc has been found to show small variations for different types of proteins, and values of about 0.18ml/g have been measured, and applied, within a broad concentration range for several other proteins (40). This value was therefore chosen as a reasonable approximation for salivary proteins. Knowledge of the partial specific volume (ν) and the ratio of the molar weight to the molar refractivity (M/A) of the adsorbate permits the adsorbed amount per unit area to be calculated by applying another model (41). The values of ν and M/A employed here, 0.75ml/g and 4.10 g/ml, respectively, are representative for proteins, and have previously been used in studies of salivary protein adsorption (42, 43). For the sake of comparison, both models were applied (although only the results from the model according to DE FEIJTER *et al.* (40) are shown) giving qualitatively the same results. Minor quantitative discrepancies can be ascribed to the choice of approximate values of ν, M/A and dn/dc.

Results

Go to: Choose Go

Incubation of *A. naeslundii, S. sanguis, C. albicans* and *S. mutans* with 1U/ml Krillase for up to 75min had no effect on the bacterial viability, the counts of viable cells in the suspension remaining the same as in the enzyme-free controls (results not shown).

Presence of Krillase decreased the adhesion of *A. naeslundii, S. mitis* and *S. sanguis* to SHA beads by ≥70% (Fig. 1). The highest binding to the beads was observed in the controls, and the binding increased with the incubation time. The decrease in adherent cells varied with the strain, and most of the effect was reached within 15min.

Detachment of oral bacteria from SHA beads by Krillase depended on the incubation time (Fig.2) and enzymatic activity (Fig.3). The higher the Krillase activity, the shorter the time needed to remove bound cells. The rate of removal for the three strains tested seemed to be similar (Fig.3). In general, 1.0U/ml Krillase removed approximately 50% of the cells within 5min under the present experimental conditions.

Fig.4 shows the effect of Krillase on *in vitro* plaque as observed by SEM. Compared to the enzyme-free control, Krillase reduced the adhered microorganisms, the effect being dependent on the enzyme activity of the solution. Most of the bacteria in the artificial plaque were rod-shaped, as can be seen in the image captured at higher magnification.

The formation and enzymatic removal of *in vitro* plaque were examined with surfaces of various wettabilities. The results (Fig.5) showed that the lower the water contact angle of the surface, the denser the bacterial accumulation. No major influence by the surface wettability could, however, be observed on the plaque removal by Krillase.

The release of bacteria from denture plaque (Fig.6) proceeded with a rate of about 10^5 cells/min in the presence of 1 U/ml Krillase. This rate appeared to be constant within the time period tested (20 min). For heat-inactivated Krillase, a much slower detachment rate was found (Fig.6).

In order to collect information on the mechanisms for the enzymatic effect on plaque removal, the formation and subsequent enzymatic degradation of a salivary pellicle by Krillase on hydrophilic silica was monitored *in situ* by ellipsometry. The kinetics of the amount of protein adsorbed and the changes in adsorbed layer thickness are shown in Fig.7. The initial adsorption of salivary proteins was fast, and the adsorbed amount reached a plateau value of about 5 mg/m^2 after less than 1 h. Rinsing with buffer (at 60 min) removed reversibly bound proteins, and the adsorbed amount as well as the thickness decreased accordingly and reached new plateau values. Addition of Krillase (at 95 min) induced a gradual decrease in adsorbed material almost immediately. The process was most pronounced during the first couple of hours, then gradually slowed down. The residual adsorbed amount after 14 h of degradation was 0.4 mg/m^2. This layer was not affected by the final rinsing with buffer (at 900 min). When the adsorption of Krillase alone was tested, the plateau value of the adsorbed amount to the silica surface was about 0.2 mg/m^2 (results not shown).

Discussion

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It is evident from the present data that the enzyme mixture Krillase is capable to inhibit adhesion of oral bacteria to saliva-coated surfaces and also to detach bacterial plaque formed *in vitro* or *in vivo* on these surfaces. Thus, Krillase appears to exhibit two main mechanisms whereby plaque formation can be controlled: 1) by reducing the accumulation rate of new plaque and 2) by removing existing plaque (18). Furthermore, Krillase exhibits no microbicidal effect and, thereby, it could fulfil another important criterion for plaque control chemicals, namely to maintain an undisturbed relative balance of individual microbial species in the oral ecosystem.

The mechanism behind the inhibition of plaque accumulation appears to rely on the enzymatic degradation of salivary and microbial proteins involved in adhesion-mediating interactions between microbial cells and saliva-coated surfaces. The ability of Krillase to remove a salivary film was clearly demonstrated by the ellipsometry experiments with saliva-coated silica surfaces that were exposed to Krillase solution. Ellipsometric measurements require a highly reflective and smooth substrate surface, such as the silica surface chosen in this study. Since the surface chemistry of silica and glass is virtually the same, this also enables a comparison with results obtained with glass as substrate. Upon exposure of an adsorbed protein layer to proteolytic enzymes, various effects can be anticipated, including competitive adsorption, degradation and interfacial exchange phenomena. The present experiments revealed that Krillase, on a clinically relevant time scale (min), significantly reduced the thickness and amount of salivary proteins adsorbed to the silica surface, indicating a modification of domains/peptide segments involved in bacterial attachment.

Inhibition of plaque accumulation through destruction of microbial adhesins was not shown directly in this study. However, support for such an effect was found in earlier experiments where Krillase treatment of plaque suspensions resulted in a significant decrease in microbial cell hydrophobicity and co-aggregation pattern (own unpublished data). Further support for a Krillase-dependent effect on bacterial adhesins is derived from the experiment with the denture plaque. This plaque was accumulated for 2-3 d and, thereby, it reached a thickness of several bacterial layers. Considering the plaque architecture, the action of Krillase can be assumed to occur on the superficial bacterial layer, which has to be removed before the

enzymes will degrade the next more deeply situated layer. Thus, removal of denture plaque by Krillase implies the ability of the enzyme to destroy co-aggregates of plaque bacteria, which are mainly formed by specific adhesin-receptor interactions known to bind various oral bacteria to each other (44).

The adhesion-counteracting effects of Krillase were time dependent and activity dependent in all the experiments with pure bacterial cultures and plaque formed *in vitro*. The bacterial deposits studied in the *in vitro* experiments are mainly monolayers adherent to the saliva-coated surfaces and differ from *in vivo* formed plaque. To simulate the *in vivo* situation, experiments were included with plaque formed on dentures. This plaque was also removed in a time-dependent manner, albeit at a rate that may be considered as slow from a clinical point of view. Finding the optimal Krillase activity for clinical applications may be one of the future goals. Efforts may also be focused on developing formulations of the enzyme with increased bio-availability, since the velocity of the enzymatic reaction is expected to be a function of the total surface area of the substrate and the area occupied by the enzyme (45).

Glass and silica surfaces served as simple models of a tooth surface in this study. In some experiments, the basic requirement on the model surface was a minimal surface roughness. Initial investigations were made with sintered hydroxyapatite discs, but the porous nature of this material made interpretation of the results difficult. Replacing hydroxyapatite by glass enabled distinguishing bacterial deposits from the smooth background of the glass surface in SEM analysis. Still, it must be kept in mind that the tooth surface is not as smooth as glass, and that an influence of surface roughness on the formation and retention of plaque can be anticipated (46).

Previous studies have shown that the degree of hydrophobicity of the substrate surface influences the adsorption of salivary proteins, hydrophilic surfaces giving less adsorption, both *in vitro* and *in vivo* (42, 47). Surface wettability also affects the adhesion of oral bacteria, and hydrophobic surfaces have been found to accumulate less plaque *in vitro* as well as *in vivo* (46, 48, 49). Judging from our results, it seems likely that the degree of hydrophobicity does not play a major role neither in the formation, nor the enzymatic removal, of the plaque film. Under comparable enzyme activities, a tendency was observed for more complete deposit removal from the hydrophilic glass surfaces used in the study of the influence of surface energy on *in vitro* plaque, than from those used in the study of the influence of enzymatic activity. However, this may be due to the different cleaning procedures employed for the surfaces.

In conclusion, the ability of the enzyme mixture Krillase to modify the salivary pellicle and remove adherent plaque bacteria, as well as to inhibit binding of oral microbes to saliva-coated surfaces without affecting the microbial viability, was demonstrated. Krillase has a broad spectrum of substrates, and it may constitute a potentially interesting agent for chemical plaque control. It will be a challenge to develop Krillase formulations suitable for clinical applications and further to investigate the interactions between Krillase and excipients such as surfactants and other proteins.

Acknowledgements Go to: Choose 

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Ref rences Go to: Choose 

1. Rosan B & Lamont RJ. Dental plaque formation. *Microbes Infect* 2000; **2**: 1599-

1607.
MEDLINE

2. Nyvad B. Microbial colonization of tooth surfaces. *APMIS* 1993; **101** (Suppl 32): 1-45.
MEDLINE

3. Addy M, Slayne MA, Wade WG. The formation and control of dental plaque - an overview. *J Appl Bacteriol* 1992; **73**: 269-278.
MEDLINE

4. Marsh PD & Bradshaw DJ. Dental plaque as a biofilm. *J Ind Microbiol* 1995; **15**: 169-175.
MEDLINE

5. Jensen JL, Lamkin MS, Oppenheim FG. Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions. *J Dent Res* 1992; **71**: 1569-1576.
MEDLINE

6. Tabak LA, Levine MJ, Jain NK, Bryan AR, Cohen RE, Monte LD, Zawacki S, Nancollas GH, Slomiany A, Slomiany BL. Adsorption of human salivary mucins to hydroxyapatite. *Arch Oral Biol* 1985; **30**: 423-427.
MEDLINE

7. Glantz P-O & Baier RE. Recent studies on nonspecific aspects of intraoral adhesion. *J Adhesion* 1986; **20**: 227-244.

8. Carlén A, Bratt P, Stenudd C, Olsson J, Strömberg N. Agglutinin and acidic proline-rich protein receptor patterns may modulate bacterial adherence and colonization on tooth surfaces. *J Dent Res* 1998; **77**: 81-90.
MEDLINE

9. Gibbons RJ. Bacterial adhesion to oral tissues: a model for infectious diseases. *J Dent Res* 1989; **68**: 750-760.
MEDLINE

10. Gibbons RJ. Role of adhesion in microbial colonization of host tissues: a contribution of oral microbiology. *J Dent Res* 1996; **75**: 866-870.
MEDLINE

11. Gibbons RJ & Hay DI. Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces. *Infect Immun* 1988; **56**: 439-445.
MEDLINE

12. Mombelli A. Antibiotics in periodontal therapy. In: Lindhe J, Karring T, Lang NP, eds. Clinical periodontology and implant dentistry 3rd edition. Copenhagen: Munksgaard, 1998; 488-507.

13. Steinberg D & Rothman M. Antibacterial effect of chlorhexidine on bacteria adsorbed onto experimental dental plaque. *Diagn Microbiol Infect Dis* 1996; **26**: 109-115.
MEDLINE

14. Simonsson T, Arnebrant T, Petersson L. The effect of delmopinol on salivary pellicles, the wettability of tooth surfaces *in vivo* and bacterial cell surfaces *in vitro*. *Biofouling* 1991; **3**: 251-260.

15. Rundegren J, Simonsson T, Petersson L, Hansson E. Effect of delmopinol on the cohesion of glucan-containing plaque formed by *Streptococcus mutans* in a flow cell system. *J Dent Res* 1992; **71**: 1792-1796.
MEDLINE

16. Waaler SM, Rölla G, Skjörland KK, Ögaard B. Effects of oral rinsing with

triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. *Scand J Dent Res* 1993; **101**: 192-195.
MEDLINE

17. Listgarten MA, Lindhe J, Hellden L. Effect of tetracycline and/or scaling on human periodontal disease. Clinical, microbiological, and histological obs rvations. *J Clin Periodontol* 1978; **5**: 246-271.
MEDLINE

18. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. *J Dent Res* 1992; **71**: 1431-1438.
MEDLINE

19. Moran J, Addy M, Newcombe R. Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpaste on the development of plaque and gingivitis. *J Clin Periodontol* 1989; **16**: 295-299.
MEDLINE

20. Hull PS. Chemical inhibition of plaque. *J Clin Periodontol* 1980; **7**: 431-442.
MEDLINE

21. Robinson RJ, Stoller NH, Vilardi M, Cohen DW. Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults. *Community Dent Oral Epidemiol* 1975; **3**: 271-275.
MEDLINE

22. Budtz-Jørgensen E. Prevention of denture plaque formation by an enzyme denture cleanser. *J Biol Buccale* 1977; **5**: 239-244.
MEDLINE

23. Budtz-Jørgensen E. A 3-months' study of enzymes as denture cleansers. *J Oral Rehabil* 1978; **5**: 35-39.
MEDLINE

24. Budtz-Jørgensen E, Kelstrup J, Poulsen S. Reduction of formation of denture plaque by a protease (Alcalase). *Acta Odontol Scand* 1983; **41**: 93-98.
MEDLINE

25. Kelstrup J, Funder-Nielsen TD, MØller EN. Enzymatic reduction of the colonization of *Streptococcus mutans* in human dental plaque. *Acta Odontol Scand* 1973; **31**: 249-253.
MEDLINE

26. Kelstrup J, Holm-Pedersen P, Poulsen S. Reduction of the formation of dental plaque and gingivitis in humans by crude mutanase. *Scand J Dent Res* 1978; **86**: 93-102.
MEDLINE

27. Ödman PA. The effectiveness of enzyme-containing denture cleanser. *Quintessence Int* 1992; **23**: 187-190.
MEDLINE

28. Anheller J-E, Hellgren L, Karlstam B, Vincent J. Biochemical and biological profile of a new enzyme preparation from Antarctic krill (*E. superba*) suitable for debridement of ulcerative lesions. *Arch Dermatol Res* 1989; **281**: 105-110.
MEDLINE

29. Hellgren L, Karlstam B, Mohr V, Vincent J. Peptide hydrolases from antarctic krill - an important new tool with promising medical potential. In: Margesin R, Schinner F, eds. *Biotechnological applications of cold-adapted organisms* . Berlin: Springer-Verlag, 1999; 63-74.

30. Azzam RMA & Bashara NM. *Ellipsometry and polarized light* . Amsterdam:

North-Holland, 1977.

31. Bucht A & Karlstam B. Isolation and immunological characterization of three highly purified serine proteinases from Antarctic krill (*Euphausia superba*). *Polar Biol* 1991; **11**: 495-500.
32. Karlstam B. Crossed immunoelectrophoretic analysis of proteins from Antarctic krill (*Euphausia superba*) with special reference to serine proteinases. *Polar Biol* 1991; **11**: 489-493.
33. Karlstam B, Johansson B, Brynö C. Identification of proteolytic isozymes from Antarctic krill (*Euphausia superba*) in an enzymatic debrider. *Comp Biochem Physiol* 1991; **100B**: 817-820.
34. Osnes KK. *Peptide hydrolases of Antarctic krill*, Euphausia superba. Doctoral thesis, Norwegian Institute of Technology, Trondheim, Norway, 1985.
35. Elwing H, Welin S, Askendal A, Nilsson U, Lundström I. A wettability gradient method for studies of macromolecular interactions at the liquid/solid interface. *J Colloid Interface Sci* 1987; **119**: 203-210.
36. Wannerberger K, Welin-Klintström S, Arnebrant T. Activity and adsorption of lipase from *Humicola lanuginosa* on surfaces with different wettabilities. *Langmuir* 1997; **13**: 784-790.
[redacted]
37. Wahlgren M & Arnebrant T. Adsorption of β-lactoglobulin onto silica, methylated silica, and polysulfone. *J Colloid Interface Sci* 1990; **136**: 259-265.
38. Landgren M & Jönsson B. Determination of the optical properties of Si/SiO_2 surfaces by means of ellipsometry, using different ambient media. *J Phys Chem* 1993; **97**: 1656-1660.
39. Dawes C. Rhythms in salivary flow rate and composition. *Int J Chronobiol* 1974; **2**: 253-279.
[redacted]
40. De Feijter JA, Benjamins J, Veer FA. Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air-water interface. *Biopolymers* 1978; **17**: 1759-1772.
41. Cuypers PA, Corsel JW, Janssen MP, Kop JMM, Hermens WT, Hemker HC. The adsorption of prothrombin to phosphatitylserine multilayers quantitated by ellipsometry. *J Biol Chem* 1983; **258**: 2426-2431.
[redacted]
42. Vassilakos N, Arnebrant T, Glantz P-O. Adsorption of whole saliva onto hydrophilic and hydrophobic solid surfaces. The influence of concentration, ionic strength and pH. *Scand J Dent Res* 1992; **100**: 346-353.
[redacted]
43. Lindh L, Arnebrant T, Isberg P-E, Glantz P-O. Concentration dependence of adsorption from human whole resting saliva at solid/liquid interfaces: an ellipsometric study. *Biofouling* 1999; **14**: 189-196.
44. Kolenbrander PE & London J. Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 1993; **175**: 3247-3252.
[redacted]
45. Mosbach K. Enzymes and general biology: special reference to enzyme synthesis, purification, and reactions on matrixes. *J Dent Res* 1972; **51**: 217-223.
[redacted]
46. Quirynen M & Bollen CML. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol* 1995; **22**: 1-14.
[redacted]

47. Vassilakos N, Glantz P-O, Arnebrant T. Reflectometry: a new method for quantitative determinations of intraoral film formation. *Scand J Dent Res* 1993; **101**: 339-343.
MEDLINE

48. Glantz P-O. *On wettability and adhesiveness*. Doctoral thesis, Lund University, Lund, Sweden, 1969.

49. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Arends J, Darius PL, van Steenberghe D. The influence of surface-free energy on planimetric plaque growth in man. *J Dent Res* 1989; **68**: 796-799.
MEDLINE

In the United States Patent and Trademark Office

In re: deFaire		BOARD OF PATENT APPEALS AND INTERFERENCES
Serial No.:	09/549,642	
Filing Date:	April 14, 2000	
For:	REMOVING DENTAL PLAQUE WITH KRILL ENZYMES	PATENT APPEAL
Docket No.:	314572-101F	
Art Unit:	1642	
Examiner:	Brumback, B.	

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APPELLANT'S APPEAL BRIEF

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On the Brief

TABLE OF CONTENTS

(1) **Real Party in Interest**

The inventors have assigned their interest to Phairson Medical Inc. is the real party in interest.

(2) **Related Appeals and Interferences**

On information and belief, there are no other appeals or interferences that will directly affect or have a bearing on the Board's decision in this Appeal.

(3) **Status of the Claims**

Claims 142-144 are in the application. All other claims have been cancelled. All of the pending claims are subject to one or more rejections under 35 U.S.C. §§102(b), 103(a) and 112. Hence, claims 142 through 144 are appealed.

(4) **Status of Amendments Filed After Final**

There are no amendments after final. The pending claims are attached as Appendix A.

(5) **Summary of Invention**

The invention rests on the observation that the hydrolases extracted from Krill are very effective in removing plaque. Such an extract was known to be useful as a cleaning agent – but plenty of enzymes used in cleaning are ineffective to remove dental plaque.

(6) **Issues**

The issues are:

(i) ***Issue 1:*** Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?

(ii) ***Issue 2***: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

(iii) ***Issue 3***: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

(iv) *Issue 4*: Are the phrases "wherein the dental plaque is visually observable" in claim 143 and "until dental plaque is not longer visually observable" in claim 144 supported in the specification?

(7) **Grouping of claims**

The three claims in this application each raise separate issues with respect to one or more of the issues, and shall therefor be argued separately. Accordingly, there is no grouping of the claims.

(8) **Argument**

(i) *Issue 1*: **Are claims 142-144 anticipated under 35 U.S.C. §102(b), based on Hellgren et al., US Patent 4,963,491 ("Hellgren")?**

The rejection under 35 U.S.C. §102(b) is premised on principles of inherent anticipation, since an electronic word search of the cited document confirms that not one word relates to any type of plaque. As discussed in M.P.E.P. §2112, such a rejection requires that the element not expressly set forth in the reference necessarily flows from the disclosures of the reference. This requirement is not met here.

The 'Hellgren patent is – in its core discussions – about cleaning wounds and objects such as textiles. One word in the specification mentions that teeth can be cleaned (at 1:31[1]). There is no concrete exemplification from which to infer what type of teeth are to be treated, or even that teeth *in situ* are to be treated. Teeth cleaning occurs often without plaque being effectively removed, or without plaque being present. Thus, treating to effectively remove plaque, as claimed, does not necessarily follow from the brief mention of teeth cleaning in the Hellgren patent.

The most recent Office Action asserts that contacting teeth with the enzymes recited necessarily removes dental plaque. In a sense this is true: If you picked the right amount and

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[1] Column 1, line 31.

application of the enzymes, if you picked teeth that had plaque, you would effectively remove dental plaque as illustrated in the Examples of Appellant's specification. However, the prior art disclosure must teach one what the effective conditions are and what to treat: the cited Hellgren patent does not. Thus, this would only necessarily follow if you made many choices not taught by Hellgren and not necessarily following from Hellgren. Thus, Appellant would submit that this compilation of untaught selection is not what is required to satisfy the "necessarily" standard. Hellgren teaches nothing at all about how much enzyme composition to apply, and does not even teach applying the treatment to teeth that have plaque. So for anticipation, Hellgren is clearly lacking that which would indicate that its methodology (of which, for this dental application, there is none) would necessarily achieve removal of plaque. The rejection is simply not supported by the cited document and should be withdrawn.

The rejection has been extended to claim 143, but nowhere do the Office Actions describe how the cited art teaches selecting subjects where "the dental plaque is visually observable." As the complete disclosure of the cited document is described above, it should be clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

The rejection has been further extended to claim 144, and again the Office Actions do not describe how the cited art teaches that the contacting "is conducted or repeated until dental plaque is not longer visually observable." Since the complete disclosure of the cited document is described above, again it is clear that no such teaching exists. Accordingly, the Examiner should be directed to withdraw this rejection.

(ii) *Issue 2*: Are claims 142-144 obvious under 35 U.S.C. §103(a), based on Hellgren?

Applicants respectfully submit that the disclosure in Hellgren that krill enzymes can be used to debride wounds or clean textiles does not render the claims obvious. While the results obtained in the debriding and textile cleaning context may be strong, nonetheless enzymes have been used in cleaning textiles for quite some time, and similarly there is a long history of use in

wound debridement. Thus, effectiveness in a different, highly complex environment, namely dental plaque, would not have been apparent to one of ordinary skill.

The complexity of dental plaque is illustrated by the following discussion in US Patent 6,159,447 (a patent which relates to treating plaque with an anchored enzyme):

> Plaque is a heterogeneous mixture of bacterial aggregations embedded in a sticky matrix. While bacterial composition of plaque ranges from 50 to 70 percent, the matrix is derived from dead cells, salivary glycoproteins and serum proteins that are laid on a polysaccharide backbone. The bacteria synthesize the polysaccharides for the plaque backbone as a step in their own colonization process. In addition to the viable bacteria and the matrix, plaque also contains food debris, small numbers of epithelial cells, white blood cells and various other components which are derived from the host and the host's activities.
>
> The formation and development or proliferation of plaque occurs in two stages. The first step may require a base layer of salivary glycoproteins on the tooth's surface as well as on the soft tissue in the oral cavity. This base organic layer, derived from saliva, is adsorbed onto the surface and forms an acquired pellicle. This insoluble acquired pellicle serves as the foundation for supragingival plaque. The second step is the bacterial colonization by "pioneering" bacteria of the acquired pellicle. Once the bacteria have attached to the surface of a structure, they aggregate, develop colonies and plague begins to form.
>
> There are well over 100 different bacterial species in various dental plaques. This variation in the types of bacteria is influenced by diet, salivary components and bacterial interactions, to name a few. The location of the plaque in the oral cavity, the time of the day, age of the patient and the status of the general oral hygiene of the patient all contribute to the implications and consequences of dental plaque and periodontal disease. Consequently, it is not surprising that plaque is a heterogeneous collection of bacterial communities attached to the tooth providing a vast array of biochemical and physiological consequences. Two major pathological conditions as consequences are periodontal disease and dental caries.

> Enzymes as therapeutic agents present unique possibilities. However, some of the early oral pathology research using enzymes was based on the assumption that they would be bactericidal to colonies of organisms found in plaque and therefore would act as "disinfectants". This approach, however, was not fruitful. Recently, it was shown that treatment of buccal epithelial cells with protease altered bacterial adhesion; however, this treatment also distorted the ratios of various bacterial populations. More promising results were obtained when the focus was shifted from bactericidal action to altering plaque formation. These latter results were seen in vitro and in vivo as well as in animal models and humans in clinical trials. However, these approaches also fell short of desired therapeutic effectiveness most likely because the required time for an effective action exceeded the retention time of the enzyme in the oral cavity. In short, salivary flow, other fluid and food movement and normal mechanical agitation in the oral cavity reduced the retention time of the enzyme(s). These factors shortened the residence time of the enzymes, resulting in less than desirable clinical efficacy.

Thus, the above discussion indicates not just that dental plaque is complex, but that other enzymes have apparently failed in removing dental plaque. Further, the publication abstracts attached as Exhibits A-C indicate further that enzyme cleaners can fail to reduce plaque. Accordingly, the cited Hellgren document describes using the enzyme to clean teeth – it does not motivate experimenting to find conditions effective to remove plaque.

Applicant submits that the fact that the type of enzyme mixture recited in the claims would be effective in removing plaque, as illustrated in Examples 33 and 34, and further illustrated in Exhibit D, was obvious only after the Appellant presented the result. Thus, the rejection represents an inappropriate hindsight reconstruction of the invention. Since it would not be apparent that teeth cleaning with the enzymes would produce plaque removal, one would not, absent hindsight, select subjects with visibly apparent plaque so as to satisfy claim 143. It would be further less apparent to continue or repeat the treatment until "dental plaque is not longer visually observable," as required by claim 144.

Accordingly, Appellant submits that this rejection should be withdrawn.

(iii) *Issue 3*: Are claims 142-144 obvious under 35 U.S.C. §103 based on Karistam, EP 257 003, in view of Ratcliff, US 4,837,009?

Karistam is cited for substantially the same disclosure as is found in Hellgren, but perhaps further including the disclosure that krill enzymes include a hyaluronidase activity that degrades a glycoaminoglycan. Ratcliff is asserted in an Office Action to teach:

> that dental plaque is a complex extracellular matrix containing glucosaminoglycans, chondroitin sulfates, glycoproteins, and proteins. Ratcliff further teaches that degradation of these compounds retards plaque growth.

The March 29, 2001 Office Action cites for these propositions the following text italicized text from Ratcliff:

> Bacterial agglutinigation includes the conversion of sucrose to glucans and fructans by enzymes known as glycosyltransferases. These enzymes are of bacterial origin. The plaque maas [sic] becomes a complex extra cellular (of microorganisms) matrix containing sulphated glucosamineglycans, proteoglycans, glycoproteins, sugar, proteins and lipids which aid in the process of bacterial agglutination. These compounds include the presence of sulphur and become unstable in the presence of high oxygen compounds. The oxygen splits the sulphide bonds to form sulphates or SO_2.
>
> Clinical observations by the inventor have led to the conclusion that **all of these biochemical compounds are attacked to a greater or lesser extent by stabilized chlorine dioxide**. Since these compounds may be used as nutrients for bacteria, the reduction of the compounds will inhibit bacterial growth. More specifically, the stabilized chlorine dioxide oxidizes carbohydrates, chondroitin sulphates, glucosaminglycans, glycoproteins, proteins and lipids. Since these compounds arise as bacterial by products and debris from dead and dying cells, are of salivary origin and are the mechanism of agglutination of the plaque mass, their degradation/oxidation retards plaque growth.

Ratcliff at 3:62 – 4:22 (citation omitted).

Applicant would respectfully submit that Ratcliff indicates that **stabilized chlorine dioxide** attacks the complex mixture of components of plaque. This teaching has no bearing on whether or not Hellgren's composition would be effective. In fact, it teaches that what the Merck Index describes as a "strongly oxidizing" gas that "reacts violently with organic compounds" is required. The rejection is simply in error.

Accordingly, Applicant respectfully submits that the rejection is in error, and should be withdrawn.

(iv) *Issue 4*: Is the phrase "wherein the dental plaque is visually observable" in claim 143 supported in the specification?

Claims 143 and 144 stand rejected under 35 U.S.C. §112, first paragraph, based on an assertion of lack of support in the specification. This support is clear from the two relevant working examples.

The two relevant examples in the specification read:

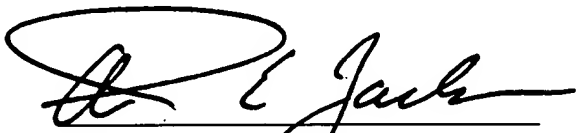
> The krill poly-enzyme preparation was used to remove dental plaque in beagles. Before use, ampoules of the poly-enzyme preparation (described in Example 1A) were reconstituted in 5 ml of saline to a final concentration of 5 Casein-Units/ml. The content from a freshly prepared ampoule was carefully painted over teeth and gingiva. The tongue was fixated for 2 minutes and food and beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily until all plaque was completely decomposed. The dogs were ***inspected*** for status of plaque, saliva secretion and adverse reactions once daily. Eight beagles with abnormal plaque formation due to special feeding and housing were included in this study. After 4 days ***all signs of plaque*** were gone and the study was terminated. No adverse reactions could be observed.
>
> The krill poly-enzyme preparation was used to remove dental plaque in humans. One ampoule of poly-enzyme preparation of Example 1A was reconstituted, in 5 ml of saline to a final concentration of 5 Casein-Units/ml, before each treatment and used to rinse the patient's mouth cavity for 5 minutes. Food and

beverage were not allowed for 2 hours post-treatment. The treatment was repeated twice daily and the patients were ***inspected once daily for plaque***, saliva secretion, dryness, and adverse reactions. The patients were not allowed to brush their teeth during the study period. The treatment was continued until ***all signs of plaque*** were gone, but not for longer than 7 days. Two hours after the first treatment all patients experienced a soft and smooth sense over their teeth but ***visual inspection showed remnants of plaque***. Two hours after the third treatment, ***all signs of plaque were gone*** and treatments were terminated. No adverse reactions were observed.

From the above, it should be quite clear that the subjects had visually apparent signs of plaque, and that appropriate treatment can be conducted to remove such visual signs. Accordingly, the specification supports the questioned recitations. Therefore the rejections should be withdrawn.

CONCLUSION

For the foregoing reasons, Appellant respectfully requests that the rejections under 35 U.S.C. §§ 102, 103 and 112 with respect to all of the pending claims be reversed and the pending claims in the application allowed.



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Dated: August 12, 2002

Appendix:

Claims on Appeal.

Exhibits:

A: Abstract of J. Clin. Periodontol 7:431-42, 1980.
B: Abstract of J. Clin. Periodontol 16:295-9, 1989.
C: Abstract of Community Dent. Oral Epidemiol. 3:271-5, 1975.
D: Berg et al., "Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control I," European J. Oral Sciences 109:316, 2001.

APPENDIX A - COPY OF CLAIMS ON APPEAL

142. A method of removing dental plaque in an animal subject comprising: contacting the dental plaque with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

143. A method of removing dental plaque comprising:
contacting the dental plaque, wherein the dental plaque is visually observable, with a dental plaque removing effective amount of a hydrolase mixture comprising enzymes from krill.

144. The method of claim 143, wherein the contacting is conducted or repeated until dental plaque is not longer visually observable.

137113.1.07

1: J Clin Periodontol 1980 Dec;7(6):431-42 Related Articles, Books, Link

Chemical inhibition of plaque.

Hull PS.

Attempts to control plaque by chemical means using enzymes, antibiotics an antiseptics are reviewed. Enzymes such as mucinase, dehydrated pancreas, enzymes of fungal origin, dextranase and mutanase showed limited clinical success despite promising in vitro and animal studies. Side effects from the of enzymes were observed. Many antibiotics have been used in attempts to control plaque and several have been successful. However, problems exist from the long-term use of such drugs which precludes their routine use as agents for controlling plaque. The biguanide chlorhexidine is the most widel used and investigated method of chemical plaque control. Many studies hav been demonstrated that it will successfully control plaque. No toxic side effects have been reported from its long-term use but local side effects such staining of the teeth do occur. The quaternary ammonium compounds have a present no advantages over the biguanides and require more frequent usage t achieve the same degree of plaque control as chlorhexidine.

Publication Types:
- Review

PMID: 7012186 [PubMed - indexed for MEDLINE]

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1: J Clin Periodontol 1989 May;16(5):295-9 Related Articles, Books, Link

Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpas on the development of plaque and gingivitis.

Moran J, Addy M, Newcombe R.

Department of Periodontology, Dental School, University of Wales College Medicine, Heath Park, Cardiff, UK.

Many toothpastes have been formulated over recent years to contain antimicrobial compounds with the aim of preventing or reducing plaque, calculus, gingival inflammation or dental caries. For many, if not all of these toothpastes, it has yet to be proven whether they are significantly better at reducing plaque and gingivitis than conventional toothpastes, for which no such therapeutic effects have been claimed. This 12-day, incomplete block designed, cross-over study compared the development of plaque and gingivi following rinsing with toothpaste slurries containing the following active ingredients: (1) hexetidine/zinc citrate, (2) 0.2% triclosan, (3) amyloglucosidase/glucose oxidase, (4) sodium fluoride/sodium monofluorophosphate (NaF, MFP). By the 8th day of the study, a significant difference in gingival crevicular fluid (GCF) and GI was found between the groups. By day 12, however, no significant difference in plaque index and gingival inflammation was found between the 4 toothpastes, although plaqu area was significantly reduced with the hexetidine/zinc citrate paste when compared to the conventional fluoride paste. It was concluded that the active ingredients added to the toothpastes evaluated in this study provided little or no more additional benefit to oral hygiene and gingival health than could be achieved with a conventional fluoride toothpaste.

Publication Types:
- Clinical Trial
- Controlled Clinical Trial

PMID: 2498397 [PubMed - indexed for MEDLINE]

1: Community Dent Oral Epidemiol 1975 Nov;3 (6):271-5

Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults.

Robinson RJ, Stoller NH, Vilardi M, Cohen DW.

One hundred and thirty-one young adults were used in a controlled study to determine whether a proteolytic mouthwash produced from B. subtilis could help remove existing plaque, decrease the rate of plaque accumulation or reduce the clinical signs of gingivitis. These subjects were stratified by mea of the Gingival Index and the Shaver-Schiff Plaque Index into severe or mil groups. These groups were further divided in a random fashion into treatme and placebo groups. The treatment group rinsed with a proteolytic mouthwa consisting mainly of neutral and alkaline proteases and amylase. A placebo mouthwash was used by the control group. The results of this investigation indicate that there is no statistically significant reduction either in existing plaque or in the rate of plaque accumulation when this proteolytic enzyme mouthwash is used. Furthermore, in the treatment group the degree of gingivitis as measured by the GI was not reduced.

Publication Types:
- Clinical Trial
- Randomized Controlled Trial

PMID: 1104247 [PubMed - indexed for MEDLINE]

Full Article

View/Print PDF article (493K)

European Journal Of Oral Sciences
Volume 109 Issue 5 Page 316 - October 2001

Proteolytic degradation of oral biofilms *in vitro* and *in vivo*: potential of proteases originating from *Euphausia superba* for plaque control

I. Cecilia Hahn Berg [1], *Sotirios Kalfas* [2,3], *Martin Malmsten* [1,4], *Thomas Arnebrant* [1,5]

This paper deals with enzymatic removal of dental plaque, *in vitro* as well as *in vivo*, using proteases from the Antarctic krill shrimp (*Euphausia superba*), referred to as Krillase®. Krillase exhibits both endo- and exopeptidase activity but has no microbicidal effect. In model systems with pure cultures of oral microorganisms, Krillase demonstrated inhibition of microbial adhesion to saliva-coated hydroxyapatite. Furthermore, a protocol for the growth of reproducible *in vitro* plaque films has been developed, and effects of Krillase on the plaque film were investigated by means of scanning electron microscopy (SEM). The results showed that Krillase efficiently released microorganisms from plaque *in vitro*, the effect being dependent on the enzymatic activity. The surface energy of the substratum had a minor influence on the formation and removal of plaque *in vitro*. Ellipsometric studies on the formation and enzymatic removal of a salivary pellicle indicated that the enzymatic effect on plaque may partly depend on degradation of the salivary pellicle. Krillase was also able to remove plaque accumulated on dentures *in vivo*. Our results demonstrate the potential of Krillase for plaque control, and that these enzymes are worthy of further investigations including clinical studies and work to find a suitable vehicle.

Accumulation of microbial plaque on tooth surfaces is the cause of caries and periodontal diseases, the most prevalent oral disorders that affect industrialised societies and account for the considerable research interest paid to the subject during the last decades. Plaque development proceeds through various phases (1-4). Initially, salivary proteins such as acidic proline-rich proteins, statherin, histatins, amylase and mucins adsorb rapidly to the solid surface and form a salivary pellicle (5, 6). The pioneer bacterial colonisers adhere to the pellicle within the next couple of hours, employing various strategies ranging from non-specific physicochemical attachment (7) to recognition of specific receptor sites at the salivary proteins and using cell surface fimbriae (8-11). Other species co-adhere with the primary colonisers, and a complex microbial community is then developed through growth and stratification of the bacteria embedded in a matrix of polymers of bacterial and salivary origin.

Control of plaque accumulation can be achieved by mechanical methods such as brushing and flossing, and by use of chemical substances such as surfactants and antibiotics (12-17). If done properly, mechanical means will give adequate results, but there are also occasions when chemical plaque control is advantageous as a complement in order to counteract plaque formation. The main disadvantage of the latter methods is that the chemicals may selectively inhibit different populations of

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bacteria, resulting in disturbances of the balance in the oral ecosystem. Emergence of resistant bacterial clones upon frequent use of antimicrobial substances is another factor limiting extensive use of some anti-plaque agents.

Enzymes with a broad spectrum of specificity for bacterial surface structures and polymers in the extracellular matrix of dental plaque have also been tried for chemical control of plaque (18). These agents usually lack the above disadvantages and have comparably low antimicrobial profile. The efficacy of enzyme formulations in removing dental or denture plaque varied in clinical studies (19-27). The substrate specificity of the enzymes tested and the administration methods employed have certainly influenced the results in the different studies.

A natural mixture of digestive proteases (referred to as Krillase®) extracted from the Antarctic krill shrimp *Euphausia superba* was earlier characterized and tested for debridement of ulcerative lesions (28, 29). Krillase consists of endo- and exopeptidases able to degrade complex proteinaceous substrates more efficiently than single-enzyme preparations. In addition, the enzymes in Krillase have naturally been selected to co-exist, which makes the autolysis rate of Krillase low. These specific properties of Krillase provided the incentive to conduct this study to examine the effect of Krillase on bacterial viability and adhesion to saliva-coated surfaces as well as the ability of Krillase to counteract formation of salivary pellicle and microbial plaque.

In order to study events at the molecular level, ellipsometry was employed. Based on the measurement of polarisation changes of light upon reflection, ellipsometry enables monitoring of adsorption/desorption processes *in situ* with a time resolution in the order of seconds (30).

Material and methods

Go to: Choose Go

Chemicals

Tris (tris[hydroxymethyl]aminomethane, ≥99% purity) and bovine serum albumin (≥96% purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). (3, 3-dimethylbutyl)dimethylchlorosilane (97% purity) was from ABCR (Karlsruhe, Germany). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). The water used in the *in vitro* plaque and ellipsometry experiments was treated by a Milli-Q Plus unit (Millipore, Bedford, MA, USA) including ion exchange, active carbon adsorption and reverse osmosis before the final 0.22- μ m filtration step, yielding ultrapure reagent-grade water of resistivity 18.2 MΩcm at 25°C. In all the other experiments, de-ionised water was used. Ethanol (99.7% pure) was from Primalco (Helsinki, Finland) and Krillase was obtained from BioPhausia (Uppsala, Sweden) (batch numbers B038912, 610008, 604040 and 710017). The lyophilised preparation was reconstituted in water (2 ml/vial) and further diluted with 0.1M Tris-HCl buffer, pH7.5, to obtain the appropriate concentration (total proteolytic activity as determined by the supplier and expressed in units per ml solution, U/ml). The characteristics of the enzyme mixture have been reported elsewhere (28, 31-34).

Effect on bacterial viability

Streptococcus mutans NCTC 10449, *Streptococcus sanguis* ATCC 10556, *Actinomyces naeslundii* ATCC 12104, and *Candida albicans* CHR (own isolate) were grown at 37°C for 24h in the presence of 5% CO_2 on *Brucella* agar (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 5% lysed blood, 0.05 mg/ml hemin and 0.01 mg/ml vitamin K. Cells harvested with a sterile cotton swab were suspended in sterile A-buffer (1mM sodium phosphate buffer containing 50 mM KCl, 1mM $CaCl_2$ and 0.1mM $MgCl_2$, pH7.0) and the optical density (at 600nm) of the suspension was adjusted to 0.5.

Cell suspension (final density 10^6-10^8 cells/ml) was mixed with Krillase solution (final activity 1 U/ml) and A-buffer to a final volume of 1 ml. In the controls, the

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Image Previews

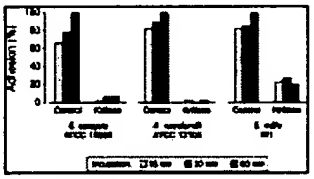

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Fig.1 Relative (%) number of bacteria adherent to saliva-coated hydroxyapatite beads at different inc...

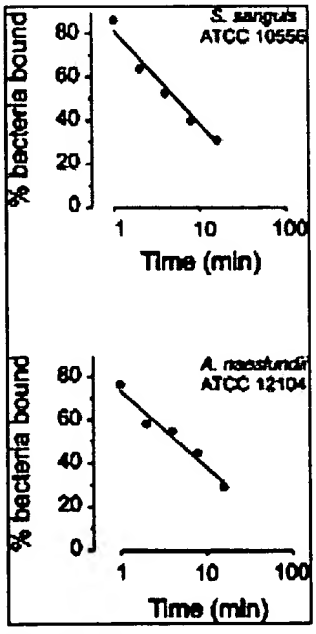

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Fig.2 Kinetics for Krillase-dependent (activity 0.5U/ml) detachment of *S. sanguis* and *A. naeslundii*... ...

Krillase solution was replaced by A-buffer. The assays were run aerobically at 37° C. Samples were taken from the mixtures after 15, 45 and 75 min of incubation and diluted serially in cold 0.1 M potassium phosphate buffer (pH6.8). From appropriate dilutions, 0.1-ml aliquots were inoculated on *Brucella* blood agar plates and incubated for 48 h at 37°C in 5% CO_2. The number of viable bacteria in the samples was determined from the number of colonies grown on the plates and is expressed in colony-forming units (CFU) per ml.

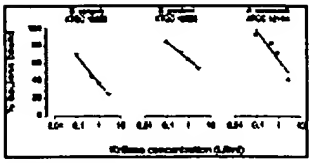

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Fig. 3 Effect of enzymatic activity on the Krillase-dependent detachment of *S. sanguis*, *S. gordonii* a...

Effect on bacterial adhesion to saliva-coated hydroxyapatite

Streptococcus mitis St1 (own isolate), *S. sanguis* ATCC 10556, *Streptococcus gordonii* ATCC 10558, and *A. naeslundii* ATCC 12104 were grown in Trypticase soy broth (Becton Dickinson) supplemented with 0.5% yeast extract (Difco, Detroit, MI, USA) and 5 µCi/ml [^{3}H]thymidine (Amersham, Little Chalfont, Bucks., England) as described above. Cells were harvested by centrifugation (6000× *g* at 6°C for 10 min) and washed twice with 5 ml A-buffer before suspended in A-buffer to yield an optical density of 2.0 at 600 nm.

Porous hydroxyapatite beads (lot number 011093-01; Clarkson Chromatography Products, South Williamsport, PA, USA) were coated with paraffin-stimulated whole saliva (SHA beads). The saliva was freshly collected on ice and clarified by centrifugation (16,000× *g*, 6°C, 10 min) before used. Alkali-washed hydroxyapatite beads were rehydrated with A-buffer overnight before incubation with saliva (40 mg beads/ml saliva) at 6°C under rocking for 1 h. The saliva was removed, and the beads were washed with 2×2 ml of A-buffer followed by a 30-min incubation with A-buffer containing 0.5% bovine serum albumin. Finally, the beads were washed with 3×3 ml of A-buffer.

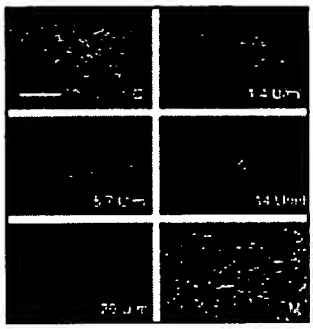

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Fig. 4 SEM images of *in vitro* plaque on glass slides incubated for 15 min in Tris-HCl buffer (control ...

Cell suspension (final density about 10^8 cells/ml) was mixed with 40 mg SHA beads and Krillase solution (final activity 1 U/ml). Sterile A-buffer was added to a total volume of 1 ml. The enzyme solution was replaced by pure A-buffer in the controls. The mixtures were incubated aerobically at 37°C under continuous rocking. After 15, 30, and 60 min of incubation, the supernating suspension was removed, and the beads were washed with 3×1 ml of A-buffer containing 0.1% formalin in order to stop the enzymatic activity and remove non-adherent cells. The beads were thereafter mixed with 10 ml of scintillation cocktail (Biosafe; Beckman, Fullerton, CA, USA) and the radioactivity from bacteria adherent to the beads was counted in a β-counter (Rackbeta; LKB Wallac, Turku, Finland). The number of bacteria adherent to the beads was calculated from the radioactivity of the samples. Relative adhesion is given as a percent of the maximum value obtained in the control mixture at 60 min.

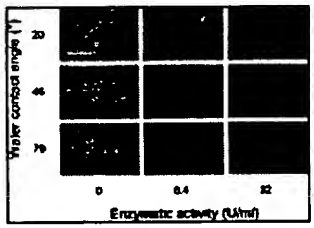

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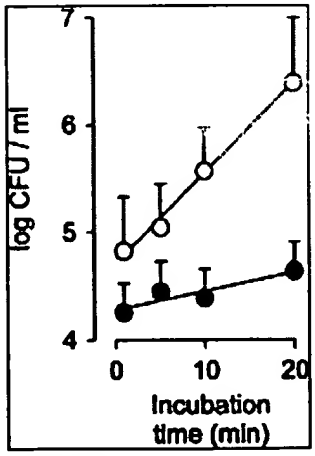
Fig. 5 SEM images of *in vitro* plaque on glass slides with various surface energy, exposed to either T...

In order to examine the effect of incubation time and enzymatic activity on the anti-adhesive properties of Krillase, cell suspension (final density about 10^8 cells/ml) was mixed with SHA beads (1 ml suspension to 200 mg beads) and incubated aerobically at 37°C for 45 min under continuous rocking. The cell suspension was removed, and the beads were washed twice with 2 ml of A-buffer in order to remove non-adherent cells. Krillase solution (0.5 ml, 0.5 U/ml) was added, and the mixture was incubated at room temperature for defined periods of time. In separate experiments, Krillase at various activities was added to the beads, and the mixture was incubated for 5 min. The assay was terminated by the addition of 1 ml 2% formalin in A-buffer. The suspension was removed, and the beads were washed twice with 2 ml A-buffer in order to remove non-adherent cells. The beads were thereafter mixed with 0.5 ml 1 M KOH and incubated for 10 min before the solution was neutralised with 1 M HCl. The beads and the liquid phase were mixed with 10 ml of scintillation cocktail, and the radioactivity was counted as described above. Relative adhesion is given as percent of the maximum value obtained in the control mixtures (no enzyme added or 0 min incubation time).



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Effect on plaque f rmed *in vitro*

In order to obtain *in vitro* plaque, saliva samples obtained after gustatory stimulation with a sugar lump were poured into polystyrene Petri dishes (about 8 ml

saliva per dish) containing glass slides ($1 \times 1 cm^2$ each) that had been rinsed with water and ethanol and air-dried. The dishes were incubated at 37°C for 48h. The plaque formed on the slides was rinsed twice with water, and excess water was absorbed carefully from the slide edges. The slides were then immersed for 15min in dishes containing warm (37°C) solution of Krillase in 0.1M Tris-HCl buffer, pH 7.5, or buffer alone (control). The samples were rinsed twice with water and air-dried. The sample surfaces were coated with a thin layer (~90nm) of Au/Pd (2 ×180s, 40mA; Balzers Sputter Coater SCD 050) and then examined with a Philips scanning electron microscope (SEM 515) operating at 15kV.

In separate experiments, the influence of surface energy of the substratum on plaque formation and removal was studied. For this purpose, the glass slides used for plaque accumulation had been boiled (80°C) for 5min in a 1:1:5 (by volume) mixture of 25% NH_3, 30% H_2O_2 and water, followed by rinsing in water and a second boiling step as above. Finally, the slides were rinsed twice with both water and ethanol. This treatment yielded hydrophilic glass surfaces with a static water contact angle of 20°, as measured by goniometry. From the hydrophilic glass surfaces, hydrophobic surfaces of two different surface energies were prepared by means of liquid phase silanisation in either p-xylene or trichloroethylene (35, 36). After rinsing in ethanol and p-xylene or trichloroethylene, the slides were immersed in either 0.1% or 0.06% solutions of (3, 3-dimethylbutyl)dimethylchlorosilane in p-xylene or trichloroethylene for 15 or 90min, respectively. The slides were finally rinsed four times with p-xylene or trichloroethylene and four times in ethanol and then stored in ethanol. These procedures yielded surfaces with static water contact angles of 46° (p-xylene solution) and 79° (trichloroethylene solution).

Effect on plaque formed *in vivo*

Fifteen subjects wearing dentures and having a clinically healthy oral mucosa participated in the study. All participants obtained detailed information about the study. The participants visited the clinic on three occasions. On the first visit, the dentures were cleaned professionally with brush, water and detergent. The participants were thereafter instructed to wear their dentures as usual for the consecutive 2d and to refrain from any denture cleansing procedure, apart from rinsing with tap water after meals to remove food remnants. On the third day, the plaque-removing ability of Krillase was tested. Each denture was immersed in 500 ml of water and kept under gentle agitation on a magnetic stirrer for 1min in order to remove saliva and loosely attached food remnants. The denture was then immersed in 120ml of Krillase solution (1 U/ml) and incubated at 25°C for up to 20 min under gentle agitation by orbital rotation at 18rpm. At defined time points, samples were aspirated and diluted with 0.1M potassium phosphate buffer containing 0.5% NaCl (pH6.8). From appropriate dilutions, 0.05-ml aliquots were inoculated on *Brucella* blood agar plates. The plates were incubated aerobically at 36°C for 48h, and the total number of colonies was counted.

One week later, the subjects visited the clinic again having allowed plaque to accumulate on the dentures for 2d as outlined above. The assay for plaque removal was repeated, but on this occasion a heat-inactivated (20min in boiling water) Krillase solution was used to serve as a negative control.

Effect on pellicle formation

Silica slides (Si/SiO_2) used for ellipsometric experiments were oxidised and cleaned as described in detail elsewhere (37). The silica surfaces were hydrophilic with a static water contact angle of <10°. Prior to usage, the slides were stored in ethanol. The silica surfaces were plasma-cleaned for 5min immediately before the ellipsometric measurement. Cleaning was performed in low-pressure residual air using a radio frequency glow discharge unit (Harrick Plasma Cleaner PDC-3XG; Harrick Scientific, Ossining, NY, USA).

Adsorption of salivary proteins to silica surfaces was followed by null ellipsometry, using an automated Rudolph Research thin film ellipsometer (type 43603-200E; Rudolph Research Analytical, Flanders, NJ, USA), controlled by a personal computer and operated at an angle of incidence of 67.7°. A xenon lamp, filtered to

Fig.6 Release kinetics of microbes from dentures immersed in Krillase solution (1 U/ml, open symbols...

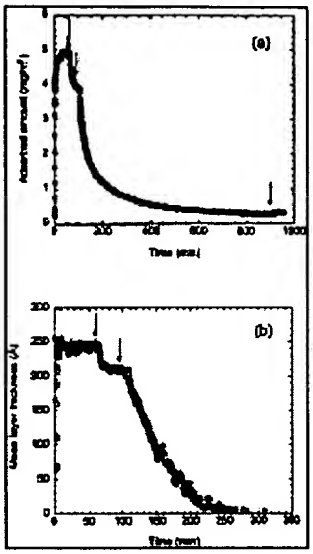

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Fig.7 Kinetics for salivary pellicle formation and enzyme-induced removal, as determined *in situ* by ...

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4015 Å, constituted the light source. The silica surface was mounted in a thermostated (25°C) quartz cuvette, agitated by a magnetic stirrer at about 90rpm. Each experiment was preceded by a four-zone calibration of the surface in two ambient media, air and buffer (10mM sodium phosphate buffer with 50mM NaCl, pH7.0), in order to determine the complex refractive index of the silicon and the refractive index and thickness of the oxide layer. A detailed description of the instrumental set-up and procedure can be found elsewhere (38). Unstimulated saliva, collected according to the procedure described by DAWES (39), was added to the buffer in the cuvette to a final concentration of 10% (v/v). The adsorption process was monitored continuously in one zone, taking into account the corrections for imperfections in optical components obtained from the four-zone calibration. In order to remove non-adsorbed salivary components, the pellicle formation was interrupted after 1h by rinsing with buffer for 35min. Krillase was then added to a final activity of 0.14U/ml, and following the degradation process (after 13h and 25min), the cuvette was finally rinsed for 45min. The flow rate used at rinsing was 1.6ml/min.

The thickness and mean refractive index of the adsorbed film are related to the output parameters of the ellipsometer, the ellipsometric angles Ψ and Δ. The adsorbed amount per unit area can be calculated (40), if the refractive index increment of the adsorbing molecules with concentration in solution (dn/dc) is known and assumed to be constant up to the concentration found in the adsorbed layer. The value of dn/dc has been found to show small variations for different types of proteins, and values of about 0.18ml/g have been measured, and applied, within a broad concentration range for several other proteins (40). This value was therefore chosen as a reasonable approximation for salivary proteins. Knowledge of the partial specific volume (ν) and the ratio of the molar weight to the molar refractivity (M/A) of the adsorbate permits the adsorbed amount per unit area to be calculated by applying another model (41). The values of ν and M/A employed here, 0.75ml/g and 4.10 g/ml, respectively, are representative for proteins, and have previously been used in studies of salivary protein adsorption (42, 43). For the sake of comparison, both models were applied (although only the results from the model according to DE FEIJTER *et al.* (40) are shown) giving qualitatively the same results. Minor quantitative discrepancies can be ascribed to the choice of approximate values of ν, M/A and dn/dc.

R sults

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Incubation of *A. naeslundii, S. sanguis, C. albicans* and *S. mutans* with 1U/ml Krillase for up to 75min had no effect on the bacterial viability, the counts of viable cells in the suspension remaining the same as in the enzyme-free controls (results not shown).

Presence of Krillase decreased the adhesion of *A. naeslundii, S. mitis* and *S. sanguis* to SHA beads by ≥70% (Fig.1). The highest binding to the beads was observed in the controls, and the binding increased with the incubation time. The decrease in adherent cells varied with the strain, and most of the effect was reached within 15min.

Detachment of oral bacteria from SHA beads by Krillase depended on the incubation time (Fig.2) and enzymatic activity (Fig.3). The higher the Krillase activity, the shorter the time needed to remove bound cells. The rate of removal for the three strains tested seemed to be similar (Fig.3). In general, 1.0U/ml Krillase removed approximately 50% of the cells within 5min under the present experimental conditions.

Fig.4 shows the effect of Krillase on *in vitro* plaque as observed by SEM. Compared to the enzyme-free control, Krillase reduced the adhered microorganisms, the effect being dependent on the enzyme activity of the solution. Most of the bacteria in the artificial plaque wer rod-shaped, as can be seen in the image captured at higher magnification.

The formation and enzymatic removal of *in vitro* plaque were examined with surfaces of various wettabilities. The results (Fig.5) showed that the lower the water contact angle of the surface, the denser the bacterial accumulation. No major influence by the surface wettability could, however, be observed on the plaque removal by Krillase.

The release of bacteria from denture plaque (Fig.6) proceeded with a rate of about 10^5 cells/min in the presence of 1 U/ml Krillase. This rate appeared to be constant within the time period tested (20 min). For heat-inactivated Krillase, a much slower detachment rate was found (Fig.6).

In order to collect information on the mechanisms for the enzymatic effect on plaque removal, the formation and subsequent enzymatic degradation of a salivary pellicle by Krillase on hydrophilic silica was monitored *in situ* by ellipsometry. The kinetics of the amount of protein adsorbed and the changes in adsorbed layer thickness are shown in Fig.7. The initial adsorption of salivary proteins was fast, and the adsorbed amount reached a plateau value of about 5 mg/m^2 after less than 1 h. Rinsing with buffer (at 60 min) removed reversibly bound proteins, and the adsorbed amount as well as the thickness decreased accordingly and reached new plateau values. Addition of Krillase (at 95 min) induced a gradual decrease in adsorbed material almost immediately. The process was most pronounced during th first couple of hours, then gradually slowed down. The residual adsorbed amount after 14 h of degradation was 0.4 mg/m^2. This layer was not affected by the final rinsing with buffer (at 900 min). When the adsorption of Krillase alone was tested, the plateau value of the adsorbed amount to the silica surface was about 0.2 mg/m^2 (results not shown).

Discussion

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It is evident from the present data that the enzyme mixture Krillase is capable to inhibit adhesion of oral bacteria to saliva-coated surfaces and also to detach bacterial plaque formed *in vitro* or *in vivo* on these surfaces. Thus, Krillase appears to exhibit two main mechanisms whereby plaque formation can be controlled: 1) by reducing the accumulation rate of new plaque and 2) by removing existing plaque (18). Furthermore, Krillase exhibits no microbicidal effect and, thereby, it could fulfil another important criterion for plaque control chemicals, namely to maintain an undisturbed relative balance of individual microbial species in the oral ecosystem.

The mechanism behind the inhibition of plaque accumulation appears to rely on the enzymatic degradation of salivary and microbial proteins involved in adhesion-mediating interactions between microbial cells and saliva-coated surfaces. The ability of Krillase to remove a salivary film was clearly demonstrated by the ellipsometry experiments with saliva-coated silica surfaces that were exposed to Krillase solution. Ellipsometric measurements require a highly reflective and smooth substrate surface, such as the silica surface chosen in this study. Since the surface chemistry of silica and glass is virtually the same, this also enables a comparison with results obtained with glass as substrate. Upon exposure of an adsorbed protein layer to proteolytic enzymes, various effects can be anticipated, including competitive adsorption, degradation and interfacial exchange phenomena. The present experiments revealed that Krillase, on a clinically relevant time scale (min), significantly reduced the thickness and amount of salivary proteins adsorbed to the silica surface, indicating a modification of domains/peptide segments involved in bacterial attachment.

Inhibition of plaque accumulation through destruction of microbial adhesins was not shown directly in this study. However, support for such an effect was found in earlier experiments where Krillase treatment of plaque suspensions resulted in a significant decrease in microbial cell hydrophobicity and co-aggregation pattern (own unpublished data). Further support for a Krillase-dependent effect on bacterial adhesins is derived from the experiment with the denture plaque. This plaque was accumulated for 2-3 d and, thereby, it reached a thickness of several bacterial layers. Considering the plaque architecture, the action of Krillase can be assumed to occur on the superficial bacterial layer, which has to be removed before the

enzymes will degrade the next more deeply situated layer. Thus, removal of denture plaque by Krillase implies the ability of the enzyme to destroy co-aggregates of plaque bacteria, which are mainly formed by specific adhesin-receptor interactions known to bind various oral bacteria to each other (44).

The adhesion-counteracting effects of Krillase were time dependent and activity dependent in all the experiments with pure bacterial cultures and plaque formed *in vitro*. The bacterial deposits studied in the *in vitro* experiments are mainly monolayers adherent to the saliva-coated surfaces and differ from *in vivo* formed plaque. To simulate the *in vivo* situation, experiments were included with plaque formed on dentures. This plaque was also removed in a time-dependent manner, albeit at a rate that may be considered as slow from a clinical point of view. Finding the optimal Krillase activity for clinical applications may be one of the future goals. Efforts may also be focused on developing formulations of the enzyme with increased bio-availability, since the velocity of the enzymatic reaction is expected to be a function of the total surface area of the substrate and the area occupied by the enzyme (45).

Glass and silica surfaces served as simple models of a tooth surface in this study. In some experiments, the basic requirement on the model surface was a minimal surface roughness. Initial investigations were made with sintered hydroxyapatite discs, but the porous nature of this material made interpretation of the results difficult. Replacing hydroxyapatite by glass enabled distinguishing bacterial deposits from the smooth background of the glass surface in SEM analysis. Still, it must be kept in mind that the tooth surface is not as smooth as glass, and that an influence of surface roughness on the formation and retention of plaque can be anticipated (46).

Previous studies have shown that the degree of hydrophobicity of the substrate surface influences the adsorption of salivary proteins, hydrophilic surfaces giving less adsorption, both *in vitro* and *in vivo* (42, 47). Surface wettability also affects the adhesion of oral bacteria, and hydrophobic surfaces have been found to accumulate less plaque *in vitro* as well as *in vivo* (46, 48, 49). Judging from our results, it seems likely that the degree of hydrophobicity does not play a major role neither in the formation, nor the enzymatic removal, of the plaque film. Under comparable enzyme activities, a tendency was observed for more complete deposit removal from the hydrophilic glass surfaces used in the study of the influence of surface energy on *in vitro* plaque, than from those used in the study of the influence of enzymatic activity. However, this may be due to the different cleaning procedures employed for the surfaces.

In conclusion, the ability of the enzyme mixture Krillase to modify the salivary pellicle and remove adherent plaque bacteria, as well as to inhibit binding of oral microbes to saliva-coated surfaces without affecting the microbial viability, was demonstrated. Krillase has a broad spectrum of substrates, and it may constitute a potentially interesting agent for chemical plaque control. It will be a challenge to develop Krillase formulations suitable for clinical applications and further to investigate the interactions between Krillase and excipients such as surfactants and other proteins.

Acknowledgements Go to: Choose

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Referenc s Go to: Choose

1. Rosan B & Lamont RJ. Dental plaque formation. *Microbes Infect* 2000; 2: 1599-

1607.
MEDLINE

2. Nyvad B. Microbial colonization of tooth surfaces. *APMIS* 1993; **101** (Suppl 32): 1-45.
MEDLINE

3. Addy M, Slayne MA, Wade WG. The formation and control of dental plaque - an overview. *J Appl Bacteriol* 1992; **73**: 269-278.
MEDLINE

4. Marsh PD & Bradshaw DJ. Dental plaque as a biofilm. *J Ind Microbiol* 1995; **15**: 169-175.
MEDLINE

5. Jensen JL, Lamkin MS, Oppenheim FG. Adsorption of human salivary proteins to hydroxyapatite: a comparison between whole saliva and glandular salivary secretions. *J Dent Res* 1992; **71**: 1569-1576.
MEDLINE

6. Tabak LA, Levine MJ, Jain NK, Bryan AR, Cohen RE, Monte LD, Zawacki S, Nancollas GH, Slomiany A, Slomiany BL. Adsorption of human salivary mucins to hydroxyapatite. *Arch Oral Biol* 1985; **30**: 423-427.
MEDLINE

7. Glantz P-O & Baier RE. Recent studies on nonspecific aspects of intraoral adhesion. *J Adhesion* 1986; **20**: 227-244.

8. Carlén A, Bratt P, Stenudd C, Olsson J, Strömberg N. Agglutinin and acidic proline-rich protein receptor patterns may modulate bacterial adherence and colonization on tooth surfaces. *J Dent Res* 1998; **77**: 81-90.
MEDLINE

9. Gibbons RJ. Bacterial adhesion to oral tissues: a model for infectious diseases. *J Dent Res* 1989; **68**: 750-760.
MEDLINE

10. Gibbons RJ. Role of adhesion in microbial colonization of host tissues: a contribution of oral microbiology. *J Dent Res* 1996; **75**: 866-870.
MEDLINE

11. Gibbons RJ & Hay DI. Human salivary acidic proline-rich proteins and statherin promote the attachment of *Actinomyces viscosus* LY7 to apatitic surfaces. *Infect Immun* 1988; **56**: 439-445.
MEDLINE

12. Mombelli A. Antibiotics in periodontal therapy. In: Lindhe J, Karring T, Lang NP, eds. Clinical periodontology and implant dentistry 3rd edition. Copenhagen: Munksgaard, 1998; 488-507.

13. Steinberg D & Rothman M. Antibacterial effect of chlorhexidine on bacteria adsorbed onto experimental dental plaque. *Diagn Microbiol Infect Dis* 1996; 25: 109-115.
MEDLINE

14. Simonsson T, Arnebrant T, Petersson L. The effect of delmopinol on salivary pellicles, the wettability of tooth surfaces *in vivo* and bacterial cell surfaces *in vitro*. *Biofouling* 1991; **3**: 251-260.

15. Rundegren J, Simonsson T, Petersson L, Hansson E. Effect of delmopinol on the cohesion of glucan-containing plaque formed by *Streptococcus mutans* in a flow cell system. *J Dent Res* 1992; **71**: 1792-1796.
MEDLINE

16. Waaler SM, Rölla G, Skjörland KK, Ögaard B. Effects of oral rinsing with

triclosan and sodium lauryl sulfate on dental plaque formation: a pilot study. *Scand J Dent Res* 1993; **101**: 192-195.
MEDLINE

17. Listgarten MA, Lindhe J, Hellden L. Effect of tetracycline and/or scaling on human periodontal disease. Clinical, microbiological, and histological observations. *J Clin Periodontol* 1978; **5**: 246-271.
MEDLINE

18. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. *J Dent Res* 1992; **71**: 1431-1438.
MEDLINE

19. Moran J, Addy M, Newcombe R. Comparison of the effect of toothpastes containing enzymes or antimicrobial compounds with a conventional fluoride toothpaste on the development of plaque and gingivitis. *J Clin Periodontol* 1989; **16**: 295-299.
MEDLINE

20. Hull PS. Chemical inhibition of plaque. *J Clin Periodontol* 1980; **7**: 431-442.
MEDLINE

21. Robinson RJ, Stoller NH, Vilardi M, Cohen DW. Clinical evaluation of the effect of a proteolytic enzyme mouthwash on plaque and gingivitis in young adults. *Community Dent Oral Epidemiol* 1975; **3**: 271-275.
MEDLINE

22. Budtz-Jørgensen E. Prevention of denture plaque formation by an enzyme denture cleanser. *J Biol Buccale* 1977; **5**: 239-244.
MEDLINE

23. Budtz-Jørgensen E. A 3-months' study of enzymes as denture cleansers. *J Oral Rehabil* 1978; **5**: 35-39.
MEDLINE

24. Budtz-Jørgensen E, Kelstrup J, Poulsen S. Reduction of formation of denture plaque by a protease (Alcalase). *Acta Odontol Scand* 1983; **41**: 93-98.
MEDLINE

25. Kelstrup J, Funder-Nielsen TD, MØller EN. Enzymatic reduction of the colonization of *Streptococcus mutans* in human dental plaque. *Acta Odontol Scand* 1973; **31**: 249-253.
MEDLINE

26. Kelstrup J, Holm-Pedersen P, Poulsen S. Reduction of the formation of dental plaque and gingivitis in humans by crude mutanase. *Scand J Dent Res* 1978; **86**: 93-102.
MEDLINE

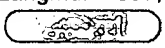
27. Ödman PA. The effectiveness of enzyme-containing denture cleanser. *Quintessence Int* 1992; **23**: 187-190.
MEDLINE

28. Anheller J-E, Hellgren L, Karlstam B, Vincent J. Biochemical and biological profile of a new enzyme preparation from Antarctic krill (*E. superba*) suitable for debridement of ulcerative lesions. *Arch Dermatol Res* 1989; **281**: 105-110.
MEDLINE

29. Hellgren L, Karlstam B, Mohr V, Vincent J. Peptide hydrolases from antarctic krill - an important new tool with promising medical potential. In: Margesin R, Schinner F, eds. *Biotechnological applications of cold-adapted organisms* . Berlin: Springer-Verlag, 1999; 63-74.

30. Azzam RMA & Bashara NM. *Ellipsometry and polarized light* . Amsterdam:

North-Holland, 1977.

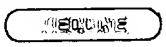
31. Bucht A & Karlstam B. Isolation and immunological characterization of three highly purified serine proteinases from Antarctic krill (*Euphausia superba*). *Polar Biol* 1991; **11**: 495-500.
32. Karlstam B. Crossed immunoelectrophoretic analysis of proteins from Antarctic krill (*Euphausia superba*) with special reference to serine proteinases. *Polar Biol* 1991; **11**: 489-493.
33. Karlstam B, Johansson B, Brynö C. Identification of proteolytic isozymes from Antarctic krill (*Euphausia superba*) in an enzymatic debrider. *Comp Biochem Physiol* 1991; **100B**: 817-820.
34. Osnes KK. *Peptide hydrolases of Antarctic krill*, Euphausia superba. Doctoral thesis, Norwegian Institute of Technology, Trondheim, Norway, 1985.
35. Elwing H, Welin S, Askendal A, Nilsson U, Lundström I. A wettability gradient method for studies of macromolecular interactions at the liquid/solid interface. *J Colloid Interface Sci* 1987; **119**: 203-210.
36. Wannerberger K, Welin-Klintström S, Arnebrant T. Activity and adsorption of lipase from *Humicola lanuginosa* on surfaces with different wettabilities. *Langmuir* 1997; **13**: 784-790.

37. Wahlgren M & Arnebrant T. Adsorption of β-lactoglobulin onto silica, methylated silica, and polysulfone. *J Colloid Interface Sci* 1990; **136**: 259-265.
38. Landgren M & Jönsson B. Determination of the optical properties of Si/SiO_2 surfaces by means of ellipsometry, using different ambient media. *J Phys Chem* 1993; **97**: 1656-1660.
39. Dawes C. Rhythms in salivary flow rate and composition. *Int J Chronobiol* 1974; **2**: 253-279.

40. De Feijter JA, Benjamins J, Veer FA. Ellipsometry as a tool to study the adsorption behavior of synthetic and biopolymers at the air-water interface. *Biopolymers* 1978; **17**: 1759-1772.
41. Cuypers PA, Corsel JW, Janssen MP, Kop JMM, Hermens WT, Hemker HC. The adsorption of prothrombin to phosphatitylserine multilayers quantitated by ellipsometry. *J Biol Chem* 1983; **258**: 2426-2431.

42. Vassilakos N, Arnebrant T, Glantz P-O. Adsorption of whole saliva onto hydrophilic and hydrophobic solid surfaces. The influence of concentration, ionic strength and pH. *Scand J Dent Res* 1992; **100**: 346-353.

43. Lindh L, Arnebrant T, Isberg P-E, Glantz P-O. Concentration dependence of adsorption from human whole resting saliva at solid/liquid interfaces: an ellipsometric study. *Biofouling* 1999; **14**: 189-196.
44. Kolenbrander PE & London J. Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol* 1993; **175**: 3247-3252.

45. Mosbach K. Enzymes and general biology: special reference to enzyme synthesis, purification, and reactions on matrixes. *J Dent Res* 1972; **51**: 217-223.

46. Quirynen M & Bollen CML. The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol* 1995; **22**: 1-14.


47. Vassilakos N, Glantz P-O, Arnebrant T. Reflectometry: a new method for quantitative determinations of intraoral film formation. *Scand J Dent Res* 1993; **101**: 339-343.
MEDLINE

48. Glantz P-O. *On wettability and adhesiveness*. Doctoral thesis, Lund University, Lund, Sweden, 1969.

49. Quirynen M, Marechal M, Busscher HJ, Weerkamp AH, Arends J, Darius PL, van Steenberghe D. The influence of surface-free energy on planimetric plaque growth in man. *J Dent Res* 1989; **68**: 796-799.
MEDLINE